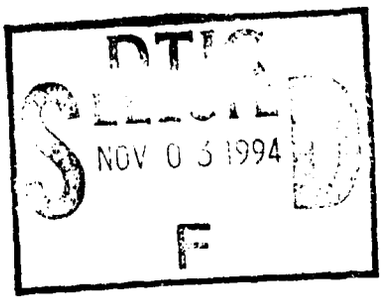

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We have used the protein synthesis inhibitor ricin and the sodium channel blocker saxitoxin (STX) as model toxic agents to investigate the feasibility of developing safe and effective vaccines against the in vivo toxicity of biological and chemical toxins. Because of their extreme in vivo toxicity, they can not be utilized as immunogens to elicit protective immunity. Thus, we have focused on the anti-idiotype and synthetic peptide-based approaches in our vaccine development strategy. A number of anti-idiotype reagents were produced, some of which were demonstrated to elicit in vivo protective immunity against ricin intoxication. In addition, recent results suggested that a cyclic ricin A peptide homologous to residues 38-112 provided some protection against ricin toxicity in vivo. The results obtained thus far with the STX system were less encouraging. Although anti-idiotype reagents produced were capable of inducing specific and systemic anti-STX antibody responses in vivo, the immunity elicited did not provide significant protection against STX toxicity. However, some delay in the time between STX administration and death was observed with some of the anti-idiotype reagents.

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FOREWORD

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I. INTRODUCTION

The protein synthesis inhibitor **ricin**, isolated by the castor plant *Ricinus communis*, and the sodium channel blocker **saxitoxin** (STX), produced by the dinoflagellates of the genus *Gonyaulax* are among the most potent biological toxins known to man (1,2). Because of their relative low molecular weight (m.w.) and their extreme *in vivo* toxicity, they can not be safely used as immunogens in the elicitation of protective immunity against their toxicity. Our laboratory has had a long-standing interest in utilizing these biological toxins and others as model agents to assess the efficacy of the anti-idiotype-based approach in the development of safe and protective vaccines against the *in vivo* toxicity of various toxins of chemical and biological origins.

Idiotypic (Id) determinants are defined as antigenic determinants associated with the variable region of antibody molecules (3). As such, Id determinants on antibody molecules can elicit the production of specific antibodies, termed anti-Id antibodies or *Ab2*, which can be utilized to serologically define the Id. Anti-Id antibodies have been classified into three categories based on their binding specificities (4,5). Anti-Id antibodies recognizing Id determinants not associated with the binding site(s) of the anti-antigen antibodies (or *Ab1*) are referred to as *Ab2α*. Thus the binding of *Ab2α* to *Ab1* can not be inhibited by the antigen. Anti-Id antibodies whose binding to *Ab1* can be inhibited by the antigen have been divided into two groups. *Ab2β* are anti-Id antibodies that recognize combining site-associated determinants and are capable of **antigenic mimicry**, i.e. they can display similar tertiary conformation, serological characteristics and biological properties exhibited by the antigen. Thus, anti-Id antibodies of the *Ab2β* category represent the anti-Id antibodies of choice in antibody-based vaccine development strategy (4). *Ab2γ* are similar to *Ab2β* in that they also recognize binding site-associated Id determinants, and their binding to *Ab1* can be inhibited by antigens. However they do not possess antigenic mimicry. Ample experimental evidence exists in support of the ability of anti-Id antibodies to mimic antigens and to induce in naive animals specific immune responsiveness to a wide variety of antigens including bacterial, viral and parasitic organisms (6-21). Our laboratory in collaboration with researchers at USAMRIID has previously demonstrated the efficacy of the anti-Id-based vaccine approach in eliciting a systemic, active and protective immune response against the *in vivo* toxicity of the trichothecene mycotoxin T-2. A murine monoclonal antibody (mAb) specific for T-2 mycotoxin was first generated and shown to protect against the *in vitro* toxicity of T-2 utilizing the Hep-2 cell assay (22,23). The anti-T-2 mAb was used to generate syngeneic anti-Id mAbs, which upon conjugation to a protein carrier and administration into syngeneic naive mice induced a systemic and specific anti-T-2 antibody response that protected the vaccinated mice against the *in vitro* toxicity of T-2 (24). This represents the first demonstration of the use of anti-Id antibody in the induction of protective immunity against toxic compounds (reviewed in 25-Appendix A, 26-Appendix B).

This final report describes our research efforts in applying the anti-Id-based approach in the development of protective vaccines against the *in vivo* toxicity of STX and ricin. Because ricin is a protein toxin composed of two disulfide-bonded glycoprotein chains (A and B chains) whose primary amino acid sequences and three dimensional structure are known (27-29), we have also investigated synthetic peptides homologous to either the A or B chains as subunit vaccines in the elicitation of protective immune responses to ricin intoxication.

II. RESULTS

A. THE SAXITOXIN SYSTEM

We had previously generated mAbs specific for STX by immunizing BALB/C mice with STX conjugated to keyhole limpet hemocyanin (KLH), and fusing their spleen cells with the mouse myeloma NS-1 cells (30). Two anti-STX mAbs, termed S1A5 (IgM_κ) and S3E.2 ($IgG1_\kappa$), with approximate binding affinity constants of $1 \times 10^6\ M^{-1}$ were isolated and shown to be capable of displacing [^{3}H]STX binding to rat cell membranes *in vitro*. Both anti-STX mAbs also protected against the STX-induced reduction of peripheral nerve action potential in rat tibial nerve when administered *in situ*. Encouraged by these results, we proceeded to produce goat polyclonal and murine monoclonal anti-Id to S3E.2 anti-STX mAb which provided better protection against STX than S1A5 (30).

1. Anti-idiotypes to S3E.2 anti-STX monoclonal antibody

a) Polyclonal anti-idiotypes to S3E.2

S3E.2 anti-STX mAb was purified from cleared ascites by saturated ammonium sulfate (SAS) cut followed by HPLC. The purified mAb was precipitated in aluminum hydroxide and used to immunize a goat for the generation of polyclonal anti-Id antibodies. Following three i.m. immunizations with 50 μg anti-Id/injection, the goat was bled and the serum was absorbed over normal mouse immunoglobulin (NMIg) conjugated to agarose (Sigma Chemical Co., St., Louis, MO) to remove anti-isotypic and anti-allotypic antibody reactivity. Specific reactivity of the absorbed anti-Id serum to S3E.2 and not to NMIg or to other mAbs (of the same isotype but of irrelevant specificity) was assessed by enzyme-linked immunosorbent assays (ELISAs). The specific goat anti-Id antibodies were further purified through absorption to and elution from protein A chromatography, and alum precipitated. A group of five BALB/C mice were immunized intraperitoneally (i.p.) with 50 μg/injection of this anti-Id preparation every two weeks. The mice were bled before each immunization, and their sera were tested for STX reactivity in ELISA, as we previously described (30). No ELISA anti-STX reactivity was detected subsequent to the second immunization. However, following the third injection, significant ELISA titers were observed in all 5 immunized mice (Table 1). Their anti-STX antibody titers increased with subsequent immunizations, reaching titers ranging between 1:3200 to 1:12800. Following the 7th immunization with the goat anti-Id antibodies, the mice were challenged s.c. with STX and the number of dead mice and the time between STX challenge and death (elapse time) were recorded (Table 2). No significant protection against the *in vivo* toxicity of STX was observed.

b) BALB/C monoclonal anti-idiotypes to S3E.2

We have also produced anti-Id mAbs specific for S3E.2. BALB/C mice were immunized with purified S3E.2 conjugated to KLH, and alum precipitated. Anti-Id reactivity in the mouse sera was detected by a "sandwich" ELISA, as we previously described. Briefly, microliter wells were coated with 2 μg/well of purified S3E.2 and blocked with PBS (pH 7.4) supplemented with 5% normal goat serum (PBS/NGS). Serial dilutions of preimmune and immune mouse sera were added to the wells which were incubated for 1 hr at 37°C. After washing, a proper dilution of S3E.2 conjugated to biotin (31) was added to the wells. The wells were incubated for 1 hr at 37°C and washed. Anti-Id reactivity was detected by addition of avidin conjugated to horseradish peroxidase (HRP). Immune mice whose sera exhibited anti-Id activity, detected as described above, were sacrificed and their spleen cells were fused with the murine myeloma Sp2/0 for the

production of anti-Id mAbs. Screening of the fusion hybrid supernatants was done using the "sandwich" ELISA, as described above. One hybrid, designated S9-B11, was isolated that secreted an anti-Id IgM_K mAb specific for S3E.2. The binding of S9-B11 anti-Id to S3E.2 anti-STX was inhibited by STX, but not by the structurally similar toxin TDT, suggesting recognition of an Id determinant associated with the STX-binding site of S3E.2 (data not shown). These results lead us to test the ability of S9-B11 to induce in naive mice a systemic and protective anti-STX antibody response. Purified S9-B11 was conjugated to KLH and alum hydroxide-precipitated. A group of 5 BALB/C mice were vaccinated i.p. with 50 μg/injection of S9-B11-KLH every three weeks. The mice were bled before each injection and their sera tested for ELISA reactivity employing microtiter wells coated with STX-BSA (Table **3**), as described earlier (30). For *in vivo* protection experiments, a group of 19 BALB/C mice were similarly immunized with S9-B11-KLH. After the sixth injection, the mice were divided into smaller groups and were challenged s.c. with various doses of STX (Table **4**). All mice challenged with STX died, and no significant differences were noted in the elapsed time between the specific anti-Id-immune mice and that of the control groups of mice vaccinated with an anti-Id mAb of irrelevant specificity (anti-Id to anti-T-2 mycotoxin, 24).

In summary, although a specific anti-STX mAb was generated that protected against the *in vitro* toxicity of STX, neither goat polyclonal nor murine monoclonal anti-Id generated to the anti-STX mAb provided significant protection against STX *in vivo* toxicity. This may be accounted for the relative low binding affinity of S3E.2 anti-STX mAb (1×10^6 M^{-1}), although S3E.2 exhibits one of the highest binding affinity among the available anti-STX mAbs. Because of the lack of observed *in vivo* protection mediated by anti-Id reagents specific for S3E.2 anti-STX mAb, we decided to generate anti-Id reagents to a burro polyclonal anti-STX IgG preparation, generously provided by Dr. J. Hewetson, Department of Pathophysiology, USAMRIID, Fort Detrick, Frederick, MD. This burro anti-STX IgG preparation has been previously demonstrated to reverse the STX-induced cardiorespiratory failure in guinea pigs (32).

2. Anti-idiotypes to burro anti-STX IgG antibodies

a) <u>BALB/C anti-idiotypes to burro anti-STX</u>

BALB/C mice were immunized i.p. with protein G-purified goat anti-STX IgG (50 μg/injection) precipitated in aluminum hydroxide every three weeks. Following the fourth immunization, the mice were bled, their sera pooled and absorbed over a column of agarose-normal horse IgG (NHIg, Sigma Chemical Co.) until no remaining reactivity with NHIg could be detected. The reason for employing NHIg instead of normal burro Ig is the availability of the former and the cross reactivity between the two sources of normal Ig due to the close phylogenetic relationship between the two species. The absorbed mouse sera were tested for anti-Id antibody reactivity using the following ELISA. Microtiter wells were coated with 0.05 ml of a solution containing 4 μg/ml of NHIg or purified burro anti-STX IgG overnight and blocked for 1 hr. Serial dilution of the absorbed mouse sera were added, and the wells were incubated for 1 hr at 37° C and washed. Binding was detected by addition of goat anti-mouse Ig-HRP and the appropriate substrate. The results presented in Table **5** indicate that after absorption, a 1:10 dilution of the pooled mouse anti-Id serum did not react with NHIg. On the other hand, reactivity with the burro anti-STX was dilution-dependent and was still observed at a 1:10240 -1:20480 serum dilution.

The mouse anti-Id antibodies were further purified by saturated ammonium sulfate (SAS) precipitation for assessment of their ability to induce anti-STX immunity. A group of 5 BALB/C mice was vaccinated i.p. with the purified syngeneic anti-Id (50 μg/injection) every three weeks. The mice were bled before each injection, and their sera tested for anti-STX reactivity as described above (30). As reported in previous quarterly reports, no significant anti-STX antibody response was observed even after repeated vaccination (data not shown). Another group of 5 BALB/C

mice were similarly vaccinated with the purified syngeneic anti-Id antibodies conjugated to KLH (Calbiochem, San Diego, CA) using 1-ethyl-3-(3-dimehtylaminopropyl)-carbodiimide (EDAC, Sigma Chemical Co.), as previously described (31). All 5 mice vaccinated with KLH-mouse anti-Id antibodies exhibited detectable anti-STX antibody reactivity following the second injection (Table 6) (33, Appendix C). The anti-STX antibody titers did not increase significantly after subsequent booster injections, however 2/5 and 1/5 vaccinated mice developed antibody titers equal to or greater than 1/600 following the 7th and 8th injections, respectively. The anti-STX antibody response induced by syngeneic anti-Id-KLH appeared to be predominantly of the IgM isotype. Specificity of the anti-Id-induced anti-STX antibody response was demonstrated by the ability of free STX to inhibit binding. Furthermore no binding could be demonstrated to tetrodotoxin- (TDT) BSA-coated wells (33).

Anti-Id-induced anti-STX antibodies abolish the protective effect of STX in cells treated with veratridine and ouabain. Treatment of murine neuroblastoma cell line CCL131 with veratridine and ouabain results in increased ion permeability, cell swelling, and eventual death (34). In a representative experiment shown in Table 7, addition of 0.25 mM veratridine and 0.5 M ouabain resulted in approximately 77% neuroblastoma cell death (33). However, in the presence of 30 nM of STX, the veratridine/ouabain-induced cytotoxicity was reduced to ≈ 47%. When STX was preincubated with serial dilutions of pooled murine anti-STX sera, a dose-dependent decrease in the ability of STX to protect against the cytotoxicity of veratridine/ouabain was observed. Thus, a 1:10 dilution of the murine anti-STX sera significantly inhibited the protection provided by STX, and reversed veratridine/ouabain toxicity on CCL131 to 73.7% and 77.3%, respectively. No significant decrease in the STX-induced protection was observed with preimmune pooled sera (Table 7).

b) Rabbit anti-idiotypes to burro anti-STX

New Zealand white rabbits were immunized intramuscularly with the burro anti-STX IgG essentially as described above for BALB/C mice. However, our previous experience indicate that conjugation of the Ab1 to protein carrier is not required for the production of anti-Id antibodies in xenogeneic species. Therefore, unconjugated burro anti-STX IgG was used for immunization. The resulting rabbit anti-Id antibodies were rendered specific for the immunizing burro Ab1 as described for murine anti-Id above, and were purified by protein A chromatography. The specificity of the rabbit anti-Id preparation was tested using microtiter wells coated with burro anti-STX IgG or with normal horse IgG (Figure 1) (33).

To assess for the ability of the rabbit anti-Id antibodies to elicit an anti-STX antibody response a group of 5 BALB/C mice were immunized with unconjugated rabbit anti-Id i.p., as described above. The mice were bled before and after each immunization, and their sera tested for reactivity with STX (Table 8). The specificity of the murine anti-STX antibody response induced by the rabbit anti-Id was demonstrated as described above (33). Moreover, the anti-STX antibody response was capable of negating the protection afforded by STX in reversing the veratridine/ouabain toxicity on CCL131 cells (Table 9) (33).

These results prompted us to assess the ability of the murine- and rabbit-induced anti-STX antibody responses in protecting against the *in vivo* toxicity of STX. Groups of mice were immunized as above with either the syngeneic murine anti-Id-KLH or with unconjugated rabbit anti-Id antibodies. Following the 7th immunization, the mice were randomly divided into groups, and were challenged with different doses of STX subcutaneously. The time between STX administration and death, and the number of dead animals per group were recorded (Tables 10 and 11). Mice vaccinated with syngeneic anti-Id-KLH and control mice challenged with 40 μg kg STX all succumbed to STX toxicity, whereas all mice challenged with the lowest dose of STX (15 μg/kg)

survived (Table 10) (35, Appendix D). At the 30 μg/kg STX dose, one out of 10 anti-Id-vaccinated mice survived the challenge, whereas all control mice died. In addition, four of the anti-Id-vaccinated mice took longer to succumb to challenge than the controls (7:08; 8:16; 10:08 and 11:54 min. vs. 5:12 and 5:24 min.). At the 20 μg/kg STX challenge dose, only 5/10 anti-Id-vaccinated mice died. The number of control mice used in our *in vivo* experiments is usually smaller than that of experimental groups because from previous observations all control mice generally succumbed to the range of STX challenge doses used. Thus, vaccination of mice with syngeneic anti-Id-KLH was shown to induce a protective immunity against STX, at least at the 20 μg/kg STX challenge dose. On the other hand, mice immunized with rabbit anti-Id antibodies to burro anti-STX were not protected against STX challenge (Table 11). The reason for this discrepancy is not clear, however it is reasonable to suggest that lack of induced protection by the rabbit anti-Id antibodies may be due to the xenogeneic immune responses of the immunized mice to the rabbit antibodies, a situation not expected in the syngeneic mouse system.

In summary, thus far polyclonal and monoclonal anti-Id antibodies generated against our best anti-STX mAb1 (S3E.2) were able to induce systemic and specific anti-STX antibody responses (33). However, they were not effective in eliciting an antibody response capable of protecting against the *in vivo* toxicity of STX. Rabbit polyclonal anti-Id antibodies produced to the burro anti-STX IgG preparation also induced a STX-specific antibody response which did not protect the vaccinated mice against STX *in vivo* challenge. On the other hand, BALB/C mice immunized with the syngeneic mouse anti-Id antibodies conjugated to KLH were significantly protected against STX *in vivo* challenge, at least at the 20 μg/kg STX challenge dose (35).

B. THE RICIN SYSTEM

1. Passive immuno-protection against ricin intoxication

One of the objectives of the research is to generate high binding affinity anti-ricin mAbs that protect against the *in vitro* and *in vivo* toxicity of ricin. Toward this aim, a number of immunogens have been utilized, including a series of synthetic peptides homologous to either ricin chain A or chain B. The rationale for selection and the sequence of these synthetic peptides have been previously reported. Subiethal doses of native whole ricin, ricin chain A and chain B have also been used as immunogens. Among all the ricin-specific hybrids generated from a large number of fusions performed, one anti-ricin mAb was isolated that bound specifically to ricin, and protected against its *in vitro* and *in vivo* toxicity (36, Appendix E). Specificity analysis indicated that the anti-ricin mAb, termed **BG11-G2** ($IgG_{1\kappa}$), recognized an antigenic determinant whose expression requires the association of the ricin A and B chains in the native conformation of the intact ricin molecule. It exhibited no ELISA reactivity against either the isolated ricin A chain nor the ricin B chain. Moreover, it did not show any significant reactivity against T-2 mycotoxin, STX or tetrodotoxin (TDT) (36). Naive syngeneic BALB/C mice infused intraperitoneally with 10 mg of protein A-purified BG11-G2 18 to 20 hrs prior to challenge with 125 μg/kg - 250 μg/kg ricin exhibited a significant delay in the onset of death (Figure 2). When BALB/C mice were infused with 10 mg of BG11-G2 anti-ricin mAb prior to challenge, and followed by with the same amount of BG11-G2 infused 30 min, 24 hrs and 72 hrs post-challenge, a significant delay in the time of death was again observed in the group receiving 250 μg/kg ricin challenge, as compared to the control group. The group of mice that were challenged with 125 μg/kg ricin were completely protected by a combination of pre- and post-challenge infusions of BG11-G2 (Figure 3).

2. Anti-idiotypes to BG11-G2 anti-ricin mAb

a) Polyclonal anti-idiotypes to BG11-G2

i) Rabbit anti-idiotypes to BG11-G2. Because of the protection afforded by BG11-G2 anti-ricin mAb, we sought to make rabbit and goat anti-Id antibodies specific to BG11-G2. Toward this end, New Zealand White rabbits were immunized intramuscularly every three weeks with 50 μg of protein A-purified BG11-G2 precipitated in aluminum hydroxide. The first injection was administered in Freund's complete adjuvant (FCA), the second in Freund's incomplete adjuvant (FIA), and subsequent injections were given in PBS, pH 7.4. The rabbits were bled after the fourth and fifth injections and the sera were pooled. The pooled sera were adsorbed exhaustively over normal mouse Ig-Agarose chromatography until no remaining reactivity with normal mouse Ig was observed. The rabbit anti-Id IgG antibodies were further purified by protein A chromatography. To assess the ability of the rabbit anti-Id to BG11-G2 to induce an anti-ricin antibody response, a group of five BALB/C mice were immunized i.p. with 50 μg of the purified, alum-precipitated rabbit anti-Id in FCA, followed by FIA and PBS. The mice were bled before each immunization, and their sera were tested for ELISA reactivity using whole ricin-coated microtiter wells as we previously described (36). Significant anti-ricin antibody titers were not achieved following the first vaccination with rabbit anti-Id antibodies (Table **12**). However, following the second injection, anti-ricin antibodies were detected in all the vaccinated mice. The anti-ricin antibody response appears to be specific in that no significant reactivity was observed when STX-coated wells were used in the ELISA (data not shown). The vaccinated mice were challenged with various doses of ricin following the sixth injection (Table **13**). All mice challenged with 50 μg/kg ricin died from challenge within 42-69 hrs. On the other hand, off the three rabbit anti-Id-immunized mice, one mouse was protected from ricin challenge of 35 μg/kg ricin, whereas both control mice challenged with the same ricin dose succumbed. Moreover, there appeared to be a significant delay in the elapsed time between ricin administration and death in the immune group (91 and 114 hrs) vs. that in the control group (42 and 52 hrs). These results led us to similarly immunize a larger group of BALB/C mice, and to challenge them with ricin after the six immunization (Table **14**). Again, as we noted above all mice challenged with 50 μg/kg ricin died from the challenge. In this *in vivo* challenge experiment, all mice challenged with 35 μg/kg ricin also succumbed. However, in the groups that were challenged with 20 μg/kg ricin, only 2 out of 7 vaccinated mice died, whereas 2/2 control mice died (Table **14**). Thus, vaccination with rabbit anti-Id to BG11-G2 elicited in naive BALB/C mice a protective anti-ricin antibody response, although the protection was only obvious at lower challenge doses of ricin.

ii) Goat anti-idiotypes to BG11- G2. In addition to producing rabbit anti-Id antibodies, a goat was also immunized with protein A-purified BG11-G2 in aluminum hydroxide as described above for rabbit immunization, except that the dose of BG11-G2 was 100 μg per injection. The goat was bled after the fourth immunization, the sera were pooled and adsorbed over normal mouse Ig-Agarose chromatography as detailed above. Absorbed goat serum with no remaining reactivity with normal mouse Ig were adsorbed to and eluted from a protein A-Agarose column for the purpose of purifying goat IgG. A group of BALB/C mice were vaccinated with the purified goat anti-Id antibodies in aluminum hydroxide, as described above. Some anti-ricin antibody reactivity was detected in the sera of the vaccinated mice following the 3rd injection of the goat anti-Id antibodies (Table **15**). The anti-ricin antibody titers became significant following the 4th injection, and increased steadily up to the 6th immunization. Seven days after the 6th immunization, the mice were challenged with ricin, as above (Table **16**). Although no obvious protection against ricin *in vivo* toxicity was observed in this challenge experiment, a larger group of BALB/C mice were similarly vaccinated with the goat anti-Id IgG preparation, and were challenged with different doses of ricin (Table **17**). All vaccinated mice died from a ricin challenge dose of 50 μg/kg with

comparable elapsed time between ricin administration and death. At the 35 μg/kg ricin dose, all mice also succumbed, however there appeared to be a delay in the time of death in the vaccinated group as compared to that of the control group. At the lowest dose of ricin challenge (20 μg/kg), both animals in the control group died whereas 5 out of 7 anti-Id vaccinated mice survived the *in vivo* ricin challenge (Table **17**). Thus, as observed with the mice vaccinated with rabbit anti-Id antibodies to BG11-G2, mice immunized with goat anti-Id antibodies also developed an anti-ricin antibody response that completely protected them against the *in vivo* toxicity of ricin, at least at a ricin challenge dose of 20 μg/kg.

Thus, vaccination of BALB/C mice with polyclonal rabbit or goat anti-Id antibodies to BG11-G2 anti-ricin mAb elicited a systemic and specific anti-ricin antibody response which appeared to protect the vaccinated mice against a low ricin challenge dose *in vivo*.

b) Monoclonal anti-Id antibodies to BG11-G2

To produce anti-Id mAbs to BG11-G2, the latter was conjugated to KLH and used to immunize a group of BALB/C mice. The mice received 50 μg of BG11-G2-KLH per injection first in FCA, followed by FIA and PBS. Anti-Id antibody activity in the moue sera was detected using a sandwich ELISA as follows. Briefly, microtiter wells were coated with 10 μg/ml of purified BG11-G2 overnight at 4°C, washed and blocked. Serial dilutions of the mouse sera were added to the wells which were incubated for 1 hr at 37°C. After incubation, the wells were washed, and BG11-G2 conjugated to biotin was added. Anti-Id reactivity was detected by the addition of avidin-HRP and the appropriate substrate. Anti-Id antibody reactivity was detectable in the mouse sera following the third injection of BG11-G2-KLH. One week after a booster injection, the mouse spleen cells were fused with the Sp2/0 murine myeloma cell line for hybrid production. Hybrids producing anti-Id mAbs to BG11-G2 were detected using the sandwich assay just described. Seven hybridomas were thus selected with binding specificities shown in Table **18**. All the isolated anti-Id mAbs are of the IgM isotype, and they all bind to BG11-G2 anti-ricin mAb, but not to normal mouse Ig. The anti-Id mAbs generated can be divided into three categories. The first category of anti-Id is represented by NK4-E7-C7 which binds only to BG11-G2, and not to rabbit or goat anti-ricin IgG. NK9-G5-B3 anti-Id mAb represents the prototype of the second type of anti-Id which recognizes BG11-G2 and one of the polyclonal anti-ricin IgG preparation. The third category is represented by NK8-F4-F10, NK9-G6-G7, 7-D10, 8-B9 and 10-B8 mAbs which react with all three anti-ricin IgG preparations. Because internal image anti-Id antibodies generally should react not only with the immunizing anti-ricin Ab1 (BG11-G2), but also with heterologous polyclonal anti-ricin (rabbit and goat), we proceeded to produce ascites from NK8-F4-F10, NK9-G6-G7, 7-D10-F9, 8-B9-H12 and 10-B8-F10, and to purify the IgM anti-Id mAbs. Antibody purification was performed from cleared ascites by saturated ammonium sulfate precipitation followed by HPLC. The purified IgM anti-Id were conjugated to KLH, and used to immunize groups of five mice each. Mice were immunized i.p. with 50 μg/injection of anti-Id mAb-KLH first in FCA, then FIA and PBS. The mice were bled before each injection, and their sera tested for anti-ricin reactivity employing ricin-coated microtiter wells. All five anti-Id mAbs induced significant anti-ricin antibody responses subsequent to the second immunization (Tables **19-23**). Each group of vaccinated mice were divided into two groups which were challenged with 35 μg/kg and 20 μg/kg ricin doses, respectively, after the sixth immunization. All the animals immunized with NK8-F4-F10 or with NK9-G6-G7 succumbed to ricin challenge (Tables **24** and **25**). Mice vaccinated with the other three anti-Id mAbs and challenged with 35 μg/kg ricin also died (Tables **26-28**). However, at the lower challenge dose of 25 μg/kg ricin, 2 out of 3 mice immunized with 7-D10-F9 survived (Table **26**). Likewise, 1/3 mice immunized with either 8-B19-H12 or 10-B8-F10 anti-Id mAbs survived the *in vivo* ricin challenge of 25 μg/kg (Tables **27** and **28**). Because some protection was achieved, albeit only at the lower dose of ricin challenge, larger groups of BALB/C mice were vaccinated with

these anti-Id mAbs and were challenged with ricin. No significant protection against ricin *in vivo* challenge was observed in these experiments (data not shown).

3. Polyclonal anti-Id antibodies to goat anti-ricin

a) Rabbit anti-idiotypes to goat anti-ricin

We have obtained a protein G-purified goat IgG anti-ricin antibodies from Dr. J. F. Hewetson, Toxinology Division, Pathophysiology Department, USAMRIID. New Zealand White rabbits were immunized with the purified goat anti-ricin IgG precipitated in aluminum hydroxide to produce polyclonal rabbit anti-Id antibodies, as described above. Anti-isotype and anti-allotype reactivities of the polyclonal anti-Id sera were removed by repeated absorptions with agarose conjugated to normal goat Ig (NGIg), and checked for lack of reactivity against NGIg by ELISA as we previously described (36). Absorbed rabbit anti-Id sera reactive with goat anti-ricin IgG, but not with NGIg, were further purified by protein A chromatography and precipitated with aluminum hydroxide. Groups of five BALB/C mice were immunized with 10 μg/injection of alum precipitated rabbit anti-Id IgG i.m. every other week, with bleedings obtained before each bleed. The mouse sera were tested for reactivity against ricin- or STX-coated microtiter wells, and for protection against ricin cytotoxicity using the EL- 4 cell assay (37, Appendix **F**). Some anti-ricin antibody reactivity was detected following the third immunization with the rabbit anti-Id, which became more pronounced following subsequent injections (Table **29**). No reactivity against STX-BSA-coated wells was observed (data not shown).

The ability of the rabbit anti-Id-induced anti-ricin antibody response to protect against the *in vitro* toxicity of ricin was assessed using the murine EL-4 leukemic cell assay, as we described previously (36). Results of a representative experiment are shown in Table **30**. A 1:10 dilution of pooled sera from the group of mice immunized with the rabbit anti-Id antibodies completely protected EL-4 cells against ricin toxicity. Protection against ricin cytotoxicity was dose-dependent. Pooled sera (1:10) from preimmune mice and from mice immunized with a control anti-Id did not exhibited any protection against ricin toxicity.

To assess for the ability of the rabbit anti-Id to goat anti-ricin to elicit *in vivo* a protective anti-ricin antibody response, a group of BALB/C mice were immunized as described above. Following the 7th injections of the anti-Id, the mice were challenged with different doses of ricin. Although some protection against ricin toxicity was achieved, the degree of protection was not very pronounced (Table **31**). All mice challenged with ricin doses greater than 40 μg/kg ricin died. However, 6 out of 8 rabbit anti-Id-vaccinated mice challenged with 40 μg/kg ricin died somewhat later than the two control mice challenged with the same dose of ricin. At a ricin dose of 25 μg/kg ricin, none of the vaccinated mice died, whereas 1 out of two control mice died.

b) Murine anti-Id to goat anti-ricin

A similar set of experiments were performed with BALB/C anti-Id to goat anti-ricin. BALB/C mice were immunized with alum-precipitated goat anti-ricin IgG, their sera were rendered specific through absorption over NGIg-agarose, and the IgG fraction was purified as described above. A group of five BALB/C mice were then vaccinated with the purified syngeneic anti-Id conjugated to KLH. They were bled before each immunization and their sera tested for ricin reactivity by ELISA (Table **32**, and 37). As was observed with mice immunized with rabbit anti-Id antibodies, sera obtained from mice immunized with syngeneic anti-Id-KLH exhibited ELISA ricin reactivity following the second or third injection. The titer appeared to increase with subsequent immunizations. The pooled mouse sera were also capable of negating the cytotoxicity of ricin on EL-4 cells (Table **30**). A 1:10 dilution of the mouse sera preincubated with 6.25 ng/ml of ricin completely abolished ricin toxicity, whereas a 1:100 dilution afforded 39.7% protection.

For *in vivo* protection experiments, a group of 30 BABL/C mice were immunized as above. Following the 7th injection, the mice were challenged with various doses of ricin (Table 33). No apparent protection was observed since all mice succumbed to ricin challenge with approximately similar elapse time between ricin administration and death.

4. Subunit Synthetic Peptide Vaccine

a) Previous results

Because of the proteinaceous nature of the ricin molecule, and its published primary amino acid sequence and tertiary structure, we have undertaken an effort to investigate the feasibility of developing an efficacious subunit vaccine against its *in vivo* toxicity. As previously described in quarterly and final reports under contract No. DAMD-17-C-90-0051, we have selected a number of sequences homologous to either the ricin A or B chain of ricin, and have synthesized the following peptides: **A-18-39** and **A-95-120** homologous to sequences of the ricin A chain, and **B-18-50** and **B-230-257** homologous to sequences of the ricin B chain. The rationale for the selection of these sequences were previously discussed. The synthetic peptides were conjugated to KLH and were used to immunize BALB/C mice either singly or in combination using various adjuvants. The conclusions from these studies are that although the ricin synthetic peptide/KLH complexes were immunogenic and elicited specific anti-ricin antibodies, none of them or their combinations induced protective immunity. To enhance the immunogenicity of the peptides, we have selected the B-18 sequence (because of its relatively high immunogenicity) and have synthesized it on a backbone of branching lysine core, termed multiple antigenic peptide (or MAP). Peptides synthesized as MAP have been previously demonstrated to render the need for carrier proteins obsolete in generating high tittered anti-peptide and anti-native antibodies (38). Immunogenicity studies of B-18-MAP in BALB/C mice indicated that a higher titer anti-ricin antibody response could be achieved with the MAP system as compared to other adjuvants employed. However, the B-18-MAP-induced anti-ricin antibody responses did not protect mice from ricin intoxication Other ricin synthetic peptides were also synthesized as MAP, and were used to immunize BALB/C mice either singly or in combination with similar negative *in vivo* protection results. The above-mentioned results were described in details in the Final Report of contract No. DAMD-17-C-90-0051 dated April 20, 1992.

b) Universal T-helper epitope

The lack of success with the subunit synthetic peptide approach led us to investigate the potential role of a universal T-helper cell epitope identified within the hepatitis B surface antigen (**H-19-33** of HBsAg) in enhancing the titer and binding affinity of the B-18 peptide-induce anti-ricin antibody response. The sequence of the H-19 epitope of HBsAg has been described in previous reports. Two different carrier formulations, namely PAM3CYS and Palmitic acid, are also being investigated in parallel with the adjuvant properties of liposome and alum. The following synthetic complexes have been generated for immunogenicity and protection studies:

i) PAM3CYS-B-18 ricin peptide/H-19-T-helper epitope/Liposome
ii) PAM3CYS-H-19-T-helper epitope/B-18 ricin epitope/Liposome
iii) PAM3CYS-B-18-ricin peptide/Liposome
iv) Palmitic acid-B-18 ricin peptide/T-helper epitope
v) Palmitic acid-H-19 helper epitope/B-18 ricin peptide

Groups of five BALB/C mice each were immunized with the above complexes in aluminum hydroxide containing 10 μg of B-18/injection every two weeks. The mice were bled before each

immunization, and their sera tested for ricin reactivity using ricin-coated wells. The total anti-ricin antibody titers are shown in Figure **4**, and the γ-specific anti-ricin titers are presented in Figures **5** and **6**. In general, the IgG and total anti-ricin titers induced by liposome-PAM3CYS-peptides were comparable to those obtained with MAP-peptides. Moreover, the order of the H-19 T-helper epitope and B-18 ricin peptide did not appear to significantly affect the anti-ricin antibody responses. The vaccinate mice were challenged s.c. with different amounts of ricin subsequent to the 4th immunization. No significant protection against ricin intoxication was observed (Table **34**).

c) Ricin A chain residues 88-112

Discussions with Dr. Hewetson and other scientists at USAMRIID suggest that a synthetic peptide contained within sequence 90-110 of the ricin A chain may represent a good neutralizing domain against ricin intoxication. A similar A chain sequence (95-120) was independently selected by us (see above) for investigation based on its hydrophylicity index, and the presence of α -helical and β-strand structures. This A chain synthetic peptide was shown by us previously to elicit significant anti-ricin antibody titers, however the antibody response did not afford significant *in vivo* protection against ricin intoxication. This discrepancy may be due to the fact that the A chain synthetic peptide 90-110 was rendered cyclic by substitution of the two terminal a.a residues with cysteines resulting in the formation of a disulfide bridge, whereas in our synthetic peptide, no disulfide bond was introduced.

Refinement of the ricin crystallographic structure to 2.5 Å, with the assistance of molecular dynamics programs, has been performed. The major differences between this and the 2.8 Å structure which are relevant to the design of crosslinked peptides described earlier for this project are that the loop comprising residue 86-90 is displayed further from the 111-114 β strand region in the 2.5 Å than in the 2.8 Å structure, based on the α-carbon backbone tracings. The sequence of the ricin A chain 88-112 is as follows:

Asn_{88} - Ser - Ala_{90} - Tyr - Phe - Phe - His - Pro_{95} - Asp - Asn - Gln - Glu - Asp_{100} - Ala - Glu - Ala - Ile - Thr_{105} - His - Leu - Phe - Thr - Asp_{110} - Val - Gln_{112}

We have been able to display the 2.5 Å structure graphically and measure various interatomic distances for atoms in residues Asn 88 and Gln 112, as shown in Figure **7**. This has permitted a more accurate prediction of satisfactory substitutions for these residues and selection of crosslinkers which will theoretically allow a bridge between positions 88 and 112 to approximately the distance predicted by the crystal structure. A hand-drawn sketch of the expanded view of the RASWIN representation of the Gln 112 and Asn 88 orientations with individual atoms identified is shown in Figure **8**. Shown on the left is the calculated distance between the side chain Gln amide nitrogen and Asn carbonyl carbon atoms in the native structure. On the right, the (Lys 112, Cys 88) substituted sequence is shown with the projected estimates of the distances between the same two positions but including the propionyl crosslinker to establish a thioether linkage between the residues. Although the lysine side chain is likely to adopt a more extended conformation compared to the structure shown for the Gln amide moiety in the intact ricin A chain molecule (Fig. **7**), as free synthetic peptides the flexibility within this region will be increased considerably, and the introduction of the propionyl-based crosslinker may be sufficient to duplicate the distance between the chains. The synthesis and cyclization reactions have been performed as described in Scheme **I**. The immunogenic formulation also include the cyclic peptide with the PAM3CYS lipoidal derivative attached to the carboxy terminus as well as the amino terminus to vary the orientation of the peptide from the lipoidal surface after intercalation. Spacer amino acid will be included between the terminal residue and the PAM3CYS to further extend the peptide from the surface. We have synthesized the cyclic peptide attached to PAM3CYS through the amino terminus and have immunized 2 groups of BALB/C mice intraperitoneally and intra

muscularly, respectively (Table **35**) Detectable anti-ricin antibody responses were observed with all mice following two injections given i m , whereas only 2/6 mice injected i p exhibited some anti-ricin antibody reactivity. However, following the third injection, all the immunized mice showed anti-ricin antibodies although the titers observed with the group immunized i m were considerably higher than those of the group injected i p. Following the fourth injection, the mice were divided into two groups and were challenged with 35 μg/kg and 25 μg/kg ricin, respectively (Table **36**). All mice whether immunized i.p. or i.m. succumbed to a ricin challenge dose of 35 μg/kg, as did the control mice. On the other hand, whereas both control mice died from a ricin challenge dose of 20 μg/kg, 2 out of 3 and 3 out of 3 mice vaccinated i.m. and i.p., respectively, survived the challenge. These results are very encouraging as they represent the first experiment with ricin synthetic subunit vaccines that provided some protection against ricin intoxication *in vivo*. We are proceeding with vaccination of a larger group of mice to confirm these results. Experiments to assess the protective potential of cyclic ricin A peptide 88-112 synthesized on PAM3CYS through the carboxy end of the peptide are also anticipated.

III. CONCLUSIONS

In summary, we have employed STX and ricin as model toxic agents in our investigation of the feasibility of developing safe and effective vaccines against their *in vivo* toxicity. Polyclonal and monoclonal anti-Id reagents have been generated to a variety of anti-STX antibodies, and have been shown to be capable of inducing systemic and specific anti-STX antibodies *in vivo*. However, none of the anti-Id reagents produced thus far could be demonstrated to elicit protective immunity against STX *in vivo* toxicity. The lack of success is presumably due to the relatively low affinity of binding of the anti-STX antibodies used to produce the anti-Id reagents. On the other hand, some success was achieved with the ricin system. Some of the anti-Id reagents to anti-ricin antibodies discussed above were able to elicit systemic and specific anti-ricin antibody responses which were demonstrated to protect against ricin intoxication *in vivo*, although the protection was usually observed when low doses of ricin challenge were employed. We are also encouraged by the recent results of *in vivo* protection afforded by the cyclic ricin A peptide 88-112. We expect to continue our investigation into this potential ricin neutralizing domain with the hope of achieving an effective synthetic subunit vaccine against ricin intoxication.

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Table 1

Saxitoxin ELISA reactivity of BALB/C mice immunized with goat anti-Id to S3E.2

Mouse designation	Immunizations				
	3°[a]	4°	5°	6°	7°
0,0	40[b]	200	400	400	3200
0,1	20	400	800	1600	6400
1,0	80	800	1600	800	6400
0,2	40	400	800	3200	12800
2,0	80	400	800	800	6400

ELISA was performed using STX-BSA-coated microtiter wells as we previously described (30).
[a]Injections (tertiary, quaternary...)
[b]Reciprocal dilution of immune sera giving OD > 3x OD of that of preimmune sera.

Table 2

In vivo STX challenge of BALB/C mice immunized with goat anti-Id to S3E.2 anti-STX

STX challenge	Survival ratio[a]		Elapsed time (min)[b]	
(μg/kg)	Control	Immune	Control	Immune
25	0/3	0/2	2:30; 2:48; 3:13	3:25; 3;31
15	0/2	0/2	4:26; 7:30	5:28; 6:07

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. STX administration and death

Table 3

Saxitoxin ELISA reactivity of BALB/C mice immunized with S9-B11 anti-Id to S3E.2

Mouse designation	Immunizations			
	3°[a]	4°	5°	6°
0,0	20[b]	NT	200	800
0,1	80	NT	400	800
1,0	80	NT	400	1600
0,2	40	NT	800	800
2,0	80	NT	800	800

ELISA was performed using STX-BSA-coated microtiter wells as we previously described (30).
[a]Injections (tertiary, quaternary...)
[b]Reciprocal dilution of immune sera giving OD > 3x OD of that of preimmune sera.

Table 4

In vivo STX challenge of BALB/C mice immunized with S9-B11 anti-Id to S3E 2 anti-STX

STX challenge	Survival ratio[a]		Elapsed time (min)[b]	
(μg/kg)	Control	Immune	Control	Immune
50	0/2	0/6	1:22:; 1:22	1:22; 1;32 2:11; 2:15 2:15; 2:20
35	0/2	0/7	1:54; 2:09	2:12; 2:25 2:25; 2:46 3:06; 3;26; 3:48
20	0/2	0/6	3:52; 4:05	3:52; 4:12 4:19; 4:28 4:35; 5:06

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. STX administration and death

Table 5

ELISA reactivity of absorbed sera from mice vaccinated with burro anti-STX IgG

Absorbed serum dilution^{-1}	Wells coated with	
	Normal horse IgG	Burro anti-STX IgG
10	0.05[a]	1.11
20	0.03	1.13
40	0	1.03
80	0	0.99
160	0	0.86
320	0	0.79
640	0	0.78
1280	0	0.64
2560	0	0.51
5120	0	0.36
10240	0	0.23
20480	0	0.10

[a] Mean of duplicate OD 410nm values.

Table 6

STX ELISA reactivity of BALB/C mice immunized with KLH-conjugated BALB/C anti-(burro anti-STX)

Mouse designation	Immunization No.								
	2	3	4	5	6	7	8		
							Ig	γ	μ
1,0	400[a]	400	400	800	800	800	800	10	1600
0,0	200	400	400	400	800	1600	1600	10	3200
0,1	200	400	200	800	400	800	800	80	1600
2,0	200	200	400	800	800	800	1600	40	3200
0,2	200	200	200	400	400	1600	1600	10	1600

[a] Reciprocal dilutions of sera considered positive (OD > 0.1 and > 3x OD of preimmune sera). ELISA binding to STX-BSA-coated wells was done as previously described (30). A pooled serum (1:40) of mice immunized with rabbit anti-Id to an anti-T-2 mAb gave an OD of 0.03 in this assay.

Total antibodies (as detected by goat anti-mouse Ig) are given for the second to the seventh bleeds. Total antibodies (Ig), γ- and μ-specific antibodies are given for the 8th bleed, as detected by isotype-specific secondary goat anti-mouse reagents.

Table 7

Anti-STX sera induced by murine anti-Id negate the protective effect of STX in veratridine/ ouabain treated cells

Neuroblastoma cell culture conditions			Dilutions of murine anti-STX induced by mouse anti-Id			
			Preimmune	Immune		
V[a]	O[b]	STX[c]	10^{-1}	10^{-1}	50^{-1}	100^{-1}
-	-	-	1.92 ± 0.2[e]	1.95 ± 0.02	1.90 ± 0.1	ND[f]	ND
+	+	-	0.44 ± 0.02 (70.0)[g]	0.45 ± 0.01 (76.9)	0.47 ± 0.01 (75.3)	ND	ND
+	+	+	1.02 ± 0.1[h] (46.8)	1.20 ± 0.1[i] (38.5)	0.50 ± 0.1[i] (73.7)	0.67 ± 0.1[i] (64.7)	1.05 ± 0.2[i] (44.7)

[a] 0.25 mM Veratridine was used in the assay
[b] 0.5 mM ouabain was used in the assay
[c] 30 nM STX was used. This concentration usually resulted in ≈ 50% protection against cytotoxicity induced by the concentrations of V and O indicated above
[d] Reciprocal dilution of sera
[e] Mean of triplicate OD ± SD
[f] Not done
[g] Percent toxicity
[h] Significant differences at 95% confidence level (*p* values ranging from 0.0261 to 0.002)
[i] Not significant

Table 8

Saxitoxin ELISA reactivity of BALB/C mice immunized with rabbit anti-(burro anti-STX)

Mouse designation	Immunization No.						
	2	3	4	5	6		
					Ig	γ	μ
1,0	400[a]	800	800	800	1600	160	3200
0,0	200	400	400	400	800	40	1600
0,1	800	400	800	1600	1600	1280	1600
2,0	400	800	1600	1600	3200	320	3200
0,2	200	200	800	800	800	160	800

[a] See legend of Table 6.

Table 9

Anti-STX sera induced by rabbit anti-Id negate the protective effect of STX in veratridine/ouabain treated cells

Neuroblastoma cell culture conditions			Dilutions of murine anti-STX induced by rabbit anti-Id				
			Preimmune	Immune			
V[a]	O[b]	STX[c]		10^{-1}	10^{-1}	50^{-1}	100^{-1}
-	-	-	1.92 ± 0.2[e]	1.90 ± 0.10	1.85 ± 0.08	ND[f]	ND
+	+	-	0.44 ± 0.02 (70.0)[g]	0.45 ± 0.01 (76.3)	0.46 ± 0.05 (75.1)	ND	ND
+	+	+	1.02 ± 0.1[h] (46.8)	1.10 ± 0.2[i] (42.1)	0.42 ± 0.05[i] (77.3)	0.9 ± 0.06[i] (58.4)	0.9 ± 0.06[i] (51.3)

[a] 0.25 mM Veratridine was used in the assay
[b] 0.5 mM ouabain was used in the assay
[c] 30 nM STX was used. This concentration usually resulted in ≈ 50% protection against cytotoxicity induced by the concentrations of V and O indicated above
[d] Reciprocal dilution of sera
[e] Mean of triplicate OD ± SD
[f] Not done
[g] Percent toxicity
[h] Significant differences at 95% confidence level (*p* values ranging from 0.0261 to 0.002)
[i] Not significant

Table 10

In vivo STX challenge of BALB/C mice vaccinated with syngeneic anti-Id-KLH to burro anti-STX

STX challenge	Survival ratio[a]		Elapsed time (min)[b]	
(µg/kg)	Control	Immune	Control	Immune
40	0/2	0/5	3:18; 4:03	3:10; 3:14 3:18; 3:31 3:48
30	0/2	**1/10**	5:12; 5:24	4:26; 4:34 5:07; 5:32 5:54; **7:08** **8:16**; **10.08** **11:54**
20	0/2	**5/10**	6:48; 8:40	5:58; 6:18 7:25; 9:21 9:30
15	2/2	5/5	All mice survived	

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. STX administration and death

Table 11

In vivo STX challenge of BALB/C mice immunized with rabbit anti-idiotypes to burro anti-STX

STX challenge	Survival ratio[a]		Elapsed time (min)[b]	
(μg/kg)	Control	Immune	Control	Immune
50	0/2	0/3	1:50:; 2:20	1:55; 2;20 2:50
40	0/2	0/7	3:00; 4:40	2:15; 2:35 3:20; 3:45 3:50; 4;45; 4:45
30	0/2	0/8	2:59; 5:17	2:57; 3:09 3:15; 3:34 3:35; 4:34 4:50; 5:40
20	1/2	0/8	5:52	4:35; 4:42 4.47; 5:12 5:15; 5:39 6:34; 7:30
10	2/2	3/3	All Animals Survived	

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. STX administration and death

Table 12

Ricin ELISA reactivity of BALB/C mice immunized with rabbit anti-idiotypes to BG11-G2

Mouse designation	Immunizations				
	2°[a]	3°	4°	5°	6°
0,0	960[b]	1600	3200	1600	3200
0,1	960	1600	3200	3200	6400
1,0	240	1600	1600	1600	3200
0,2	1920	3200	3200	6400	6400
2,0	1600	1600	3200	3200	3200

ELISA was performed using ricin-coated microtiter wells as we previously described (36).
[a]Injections (secondary, tertiary, ...)
[b]Reciprocal dilution of immune sera giving OD > 3x OD of that of preimmune sera.

Table 13

In vivo ricin challenge of BALB/C mice immunized with rabbit anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
50	0/2	0/2	42; 42	51; 69
35	0/2	**1/3**	42; 52	**91; 114** One mouse survived

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 14

In vivo ricin challenge of BALB/C mice immunized with **rabbit** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
50	0/2	0/6	44; 44	50; 50; 50 50; 54; 93
35	0/2	0/7	69; 83	69; 74; 74; 93; 93; 122 142
20	0/2	**5/7**	124; 124	124; 124 **5 mice survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 15

ELISA reactivity of BALB/C mice immunized with goat anti-Id to BG11-G2

Mouse designation	Immunizations			
	3°[a]	4°	5°	6°
0,0	80[b]	200	800	3200
0,1	0	400	800	1600
1,0	80	80	400	3200
0,2	160	200	800	1600
2,0	40	400	1600	3200

See legend of Table 12.

Table 16

In vivo ricin challenge of BALB/C mice immunized with goat anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(µg/kg)	Control	Immune	Control	Immune
50	0/2	0/2	45; 45	52; 71
35	0/2	0/3	All the mice survived	

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 17

In vivo ricin challenge of BALB/C mice immunized with goat anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(µg/kg)	Control	Immune	Control	Immune
50	0/2	0/6	47; 47	47; 47; 47 47; 47; 52
35	0/2	0/7	52; 65	71; 71; 71; **96; 98; 119** **121**
20	0/2	**5/7**	71; 97	**118; 143** **5 mice survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 18

BALB/C monoclonal anti-idiotype antibodies to BG11-G2 anti-ricin

Anti-Id mAbs	ELISA Reactivity with			
	BG11-G2	Normal mouse Ig	Rabbit anti-ricin[a]	Goat anti-ricin[b]
NK4-E7-C7	Pos	Neg	Neg	Neg
NK9-G5-B3	Pos	Neg	Pos	Neg
Nk8-F4-F10	Pos	Neg	Pos	Pos
NK9-G6-G7	Pos	Neg	Pos	Pos
7-D10	Pos	Neg	Pos	Pos
8-B9	Pos	Neg	Pos	Pos
10-B8	Pos	Neg	Pos	Pos

[a] The rabbit anti-ricin antibodies were generated by immunizing rabbits with 1.0 μg of ricin in alum. Bleeds were obtained after the fourth immunization, and IgG antibodies were purified by protein A chromatography.
[b] The goat anti-ricin IgG was a generous gift from Dr. John F. Hewetson, Toxinology Division, Department of Pathophysiology, USAMRIID.

Table 19

Ricin ELISA reactivity of BALB/C mice immunized with **NK8-F4-F10** anti-Id mAb

Mouse designation	Immunizations								
	1°[a]			2°			3°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	60[c]	0	1280	20	0	800	80	0	3200
0,1	40	0	640	20	0	800	80	0	6400
1,0	80	0	1280	20	0	800	80	0	3200
0,2	80	0	1280	40	0	1600	80	0	3200
2,0	80	0	640	40	10	800	80	0	3200

Continued

	4°[a]			5°		
	Ig	γ	μ	Ig	γ	μ
0,0	64000	0	128000	32000	0	128000
0,1	64000	0	64000	64000	0	128000
1,0	64000	1600	128000	64000	1000	256000
0,2	64000	1600	128000	64000	2000	128000
2,0	64000	1600	64000	32000	1000	128000

[a] Primary injection, secondary,....
[b] Mean reciprocal dilution of immune sera giving O.D. > or equal to that of preimmune sera.

Table 20

Ricin ELISA reactivity of BALB/C mice immunized with NK9-G6-G7 anti-Id mAb

Mouse designation	Immunizations								
	1°a			2°			3°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	40	0	1280	200	0	1600	200	0	1600
0,1	80	0	1280	100	0	800	200	0	1600
1,0	160	0	1280	200	0	1600	200	0	1600
0,2	160	0	1280	200	0	800	200	0	3200
2,0	160	0	1280	200	0	800	400	0	6400

Continued

	4°a			5°			6°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	320	0	1600	64000	320	28000	64000	4000	256000
0,1	80	0	1600	32000	3200	128000	64000	2000	256000
1,0	160	0	3200	64000	1600	128000	128000	4000	256000
0,2	160	0	3200	32000	1600	128000	128000	8000	256000
2,0	640	0	3220	32000	12800	128000	64000	32000	256000

See legend of Table 19.

Table 21

Ricin ELISA reactivity of BALB/C mice immunized with **7-D10-F9** anti-Id mAb

Mouse designation	Immunizations								
	1^{oa}			2^{o}			3^{o}		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	1280	0	>1280	>12800	0	>12800	32000	2000	128000
0,1	1280	0	>1280	>12800	0	>12800	16000	0	128000
1,0	1280	0	>1280	>12800	0	>12800	16000	0	128000
0,2	1280	0	>1280	>12800	0	>12800	16000	0	126000
2,0	1280	0	>1280	>12800	0	>12800	16000	0	128000

Continued

	4^{oa}			5^{o}		
	Ig	γ	μ	Ig	γ	μ
0,0	32000	2000	128000	32000	0	128000
0,1	32000	0	128000	16000	0	64000
1,0	32000	0	256000	64000	2000	256000
0,2	32000	0	128000	32000	0	64000
2,0	16000	0	128000	32000	0	64000

See legend of Table 19.

Table 22

Ricin ELISA reactivity of BALB/C mice immunized with **8-B9-H12** anti-Id mAb

Mouse designation	Immunizations								
	1°a			2°			3°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	160	0	1280	32000	10	128000	8000	0	32000
0,1	320	0	640	32000	10	64000	8000	0	32000
1,0	320	0	1280	32000	0	128000	8000	0	64000
0,2	320	0	1280	32000	0	64000	8000	0	32000
2,0	320	0	1280	16000	10	128000	8000	0	64000

Continued

	4°a			5°			6°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	32000	0	>128000	16000	0	64000	64000	0	256000
0,1	32000	0	>128000	8000	0	32000	32000	0	128000
1,0	32000	0	>128000	16000	0	64000	32000	0	256000
0,2	32000	0	>128000	16000	0	64000	64000	0	256000
2,0	64000	8000	>128000	16000	0	64000	64000	0	256000

See legend of Table 19.

Table 23

Ricin ELISA reactivity of BALB/C mice immunized with **10-B8-F10** anti-Id mAb

Mouse designation	Immunizations								
	1°a			2°			3°		
	Ig	γ	μ	Ig	γ	μ	Ig	γ	μ
0,0	1280	0	>1280	12800	0	>12800	16000	0	128000
0,1	1280	0	>1280	>12800	0	>12800	32000	0	>128000
1,0	640	0	>1280	>12800	1600	>12800	32000	0	128000
0,2	>1280	0	>1280	>12800	1600	>12800	32000	0	>128000
2,0	320	0	1280	16000	10	128000	8000	0	64000

Continued

	4°a			5°		
	Ig	γ	μ	Ig	γ	μ
0,0	64000	0	128000	32000	0	256000
0,1	64000	0	128000	32000	0	256000
1,0	32000	0	128000	32000	0	256000
0,2	32000	0	128000	16000	0	128000
2,0	32000	0	128000	16000	2000	256000

See legend of Table 19.

Table 24

In vivo ricin challenge of BALB/C mice immunized with **NK8-F4-F10** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
35	0/2	0/2	40; 64	64; 88
20	0/2	0/3	64; 72	136; 136; 136

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 25

In vivo ricin challenge of BALB/C mice immunized with **NK9-G6-G7** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
35	0/2	0/2	48; 64	69; 98
20	0/2	0/3	64; 72	136; 136; 136

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 26

In vivo ricin challenge of BALB/C mice immunized with **7-D10-F9** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
35	0/2	0/2	52; 70	48; 70
20	0/2	**2/3**	143; 146	149; **2 survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 27

a

In vivo ricin challenge of BALB/C mice immunized with **8-B9-H12** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
35	0/2	0/2	69; 93	76; 99
20	0/2	**1/3**	141; 144	141; 166; **1 survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 28

In vivo ricin challenge of BALB/C mice immunized with **10-B8-F10** anti-Id to BG11-G2 anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
35	0/2	0/2	52; 71	71; 76
20	0/2	**1/3**	143; 166	149; 150 **1 survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 29

Ricin ELISA reactivity of BALB/C mice immunized with rabbit anti-Id to goat anti-ricin

Mouse designation	Immunizations									
	3°[a]	4°			5°			6°		
	Ig[b]	Ig	γ[c]	μ[d]	Ig	γ	μ	Ig	γ	μ
0,0	640[e]	640	10	ND[f]	1280	ND	320	5120	640	2560
0,1	640	640	20	ND	1280	ND	640	2560	640	1280
1,0	1280	1280	10	ND	2560	ND	1280	5120	80	2560
0,2	1280	1280	20	ND	5120	ND	2560	5120	1280	1280
2,0	1280	1280	20	ND	1280	ND	1280	5120	1280	1280

[a] Immunization number (tertiary, ...)
[b,c,d] Goat anti-mouse Ig, anti-μ-chain and anti-γ-chain were used, respectively as secondary reagents.
[e] Reciprocal dilution of sera considered positive (O.D. > 3 x O.D. of preimmune sera)
[f] Not determined.

Table 30

Anti-Id-induced anti-ricin antibody responses protect against ricin cytotoxicity

Pooled mouse sera (dilution)	Without ricin	With ricin[a]	%I[b]	%P[c]
None	65,908 ± 6,234[d]	23,167 ± 3,015	64.9	
Preimmune (10)	61,978 ± 5,923	22,768 ± 2,843	63.3	
Control:				
Mouse anti-Id immune (10)	51,642 ± 5,015	19,814 ± 2,101	61.6	
Rabbit anti-Id immune (10)	49,156 ± 4,811	21,249 ± 1,958	56.8	
Experimental:				
Mouse anti-Id immune				
(10)	59,079 ± 6,102	62,152 ± 5,384	0	100.0
(50)	63,183 ± 6,623	58,923 ± 6,013	6.7	89.4
(100)	66,287 ± 7,265	43,164 ± 5,028	34.9	44.9
(200)	59,274 ± 6,292	28,425 ± 3,102	52.0	17.9
Rabbit anti-Id immune				
(10)	67,201 ± 7,017	65,918 ± 7,016	1.9	97.0
(50)	66,871 ± 6,901	55,926 ± 6,127	16.4	74.1
(100)	69,021 ± 9,170	42,635 ± 4,561	38.2	39.7
(200)	64,102 ± 6,391	29,156 ± 4,190	54.5	13.9

[a] The final concentration of ricin used was 6.25 ng/ml.
[b] Percent inhibition of cytotoxicity
[c] Percent protection
[d] Mean cpm ± SD of triplicate determination
Control anti-Id are mouse and rabbit anti-Id to an anti-T-2 mycotoxin

Table 31

In vivo ricin challenge of BALB/C mice immunized with rabbit anti-Id to goat anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
100	0/2	0/3	23; 42	28; 42; 44
75	0/2	7/7	42; 43	42; 42; 42; 45; 47; 48 52
50	0/2	7/7	42; 50	42; 48; 48 48; 50; 65 65
40	0/2	8/8	46; 52	48; 65 **76 76; 76; 96 96; 101**
25	1/2	3/3	141 **1 survived**	**All 3 mice survived**

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 32

Ricin ELISA reactivity of BALB/C mice immunized with BALB/C anti-Id to goat anti-ricin

Mouse designation	Immunizations											
	2°[a]	3°	4°			5°			6°			
	Ig[b]	Ig	Ig	γ[c]	μ[d]	Ig	γ	μ	Ig	γ	μ	
0,0	80[e]	1280	1280	10	640	2560	20	2560	5120	10	5120	
0,1	80	1280	2560	40	1280	5120	320	2560	10240	640	5120	
1,0	10	640	2560	20	1280	5120	40	5120	5120	320	5120	
0,2	320	1280	2560	80	640	1280	160	1280	5120	320	2560	
2,0	640	640	2560	160	1280	2560	160	2560	10240	320	5120	

See Legend of Table 29.

Table 33

In vivo ricin challenge of BALB/C mice immunized with **mouse anti-Id** to goat anti-ricin

Ricin challenge	Survival ratio[a]		Elapsed time (hr)[b]	
(μg/kg)	Control	Immune	Control	Immune
150	0/2	0/5	21; 27	21; 21; 27 27; 45
100	0/2	0/8	21; 25	21; 25; 30; 30; 30; 30 45; 45
75	0/2	0/8	21, 35	25; 25; 30 30; 30; 54 54; 54
40	0/2	0/5	48; 50	48; 48; 65 65; 65
25	0/2	0/4	45; 45	65; 65; 65 76

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

Table 34

In Vivo Ricin Challenge of Mice Immunized With Ricin Synthetic Peptides

Ricin synthetic peptides	Amount of ricin (μg/mouse)			
	1.5		0.8	
	Immune	Nonimmune	Immune	Nonimmune
Pam_3Cys-Liposomes				
H19-Ricin B18	2/2[a] (41 ; 41)[b]	2/2 (41 ; 43)	3/3 (67 ; 91 ; 91)	2/2 (91 ; 91)
Ricin B18-H19	2/2 (41 ; 46)		3/3 (67 ; 67 ; 116)	
Ricin B18	2/2 (41 ; 41)		3/3 (67 ; 67 ; 101)	
Palmytic Acid-Alum				
H19-Ricin B18	2/2 (42; 42)	2/2 (24 ; 46)	3/3 (66; 67 ;70)	2/2 (45; 48)
Ricin B18-H19	1/1 (48)		3/3 (45; 66; 74)	

[a]Survival ratio (No. dead mice/total no. mice)
[b]Elapsed time in hours (time between ricin administration and death)

Table 35

ELISA reactivity of BALB/C mice immunized with **ricin A 88-112 cyclic peptide**

Mouse designation	Immunizations							
	1°[a]		2°		3°		4°	
	IM[b]	IP[c]	IM	IP	IM	IP	IM	IP
0,0	NEG	NEG	1600[d]	200	1600	200	1600	400
0,1	NEG	NEG	400	NEG	1600	200	3200	100
1,0	NEG	NEG	400	NEG	6400	400	1600	200
0,2	NEG	NEG	200	400	400	200	3200	400
2,0	NEG	NEG	400	NEG	800	100	3200	400
1,1	NEG	NEG	80	NEG	1600	100	3200	100

[a] Primary injection, secondary,....
[b,c] Intramuscular and intraperitoneal injections, respectively.
[d] Mean reciprocal dilution of immune sera giving O.D. > or equal to that of preimmune sera.

Table 36

In vivo ricin challenge of BALB/C mice immunized with **ricin A 88-112 cyclic peptide**

Ricin challenge	Survival ratio[a]			Elapsed time (hr)[b]		
(μg/kg)	Control	Immune		Control	Immune	
		IM	IP		IM	IP
35	0/2	0/2	0/3	21; 68	68; 84	68; 68; 74
20	0/2	**2/3**	**3/3**	74; 140	140	

[a] Number of mice surviving/total number of mice
[b] Time in minutes between s.c. ricin administration and death

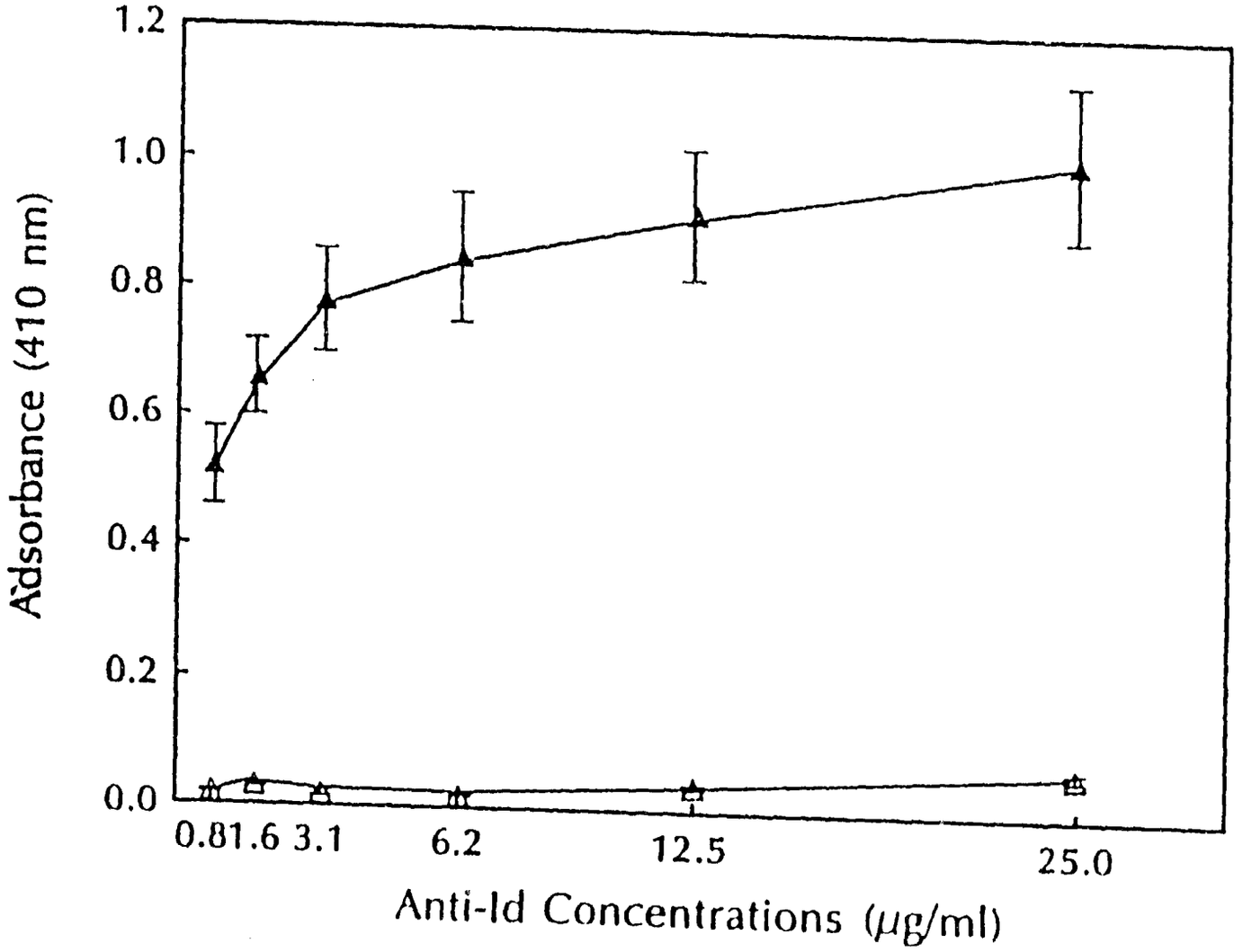


Fig. 1. ELISA reactivity of purified rabbit Ab2 anti-(burro anti-STX) using microtiter wells coated with burro anti-STX IgG (closed symbols) or with normal horse IgG (open symbols). Each point represents the mean of triplicate O.D. ± SD.

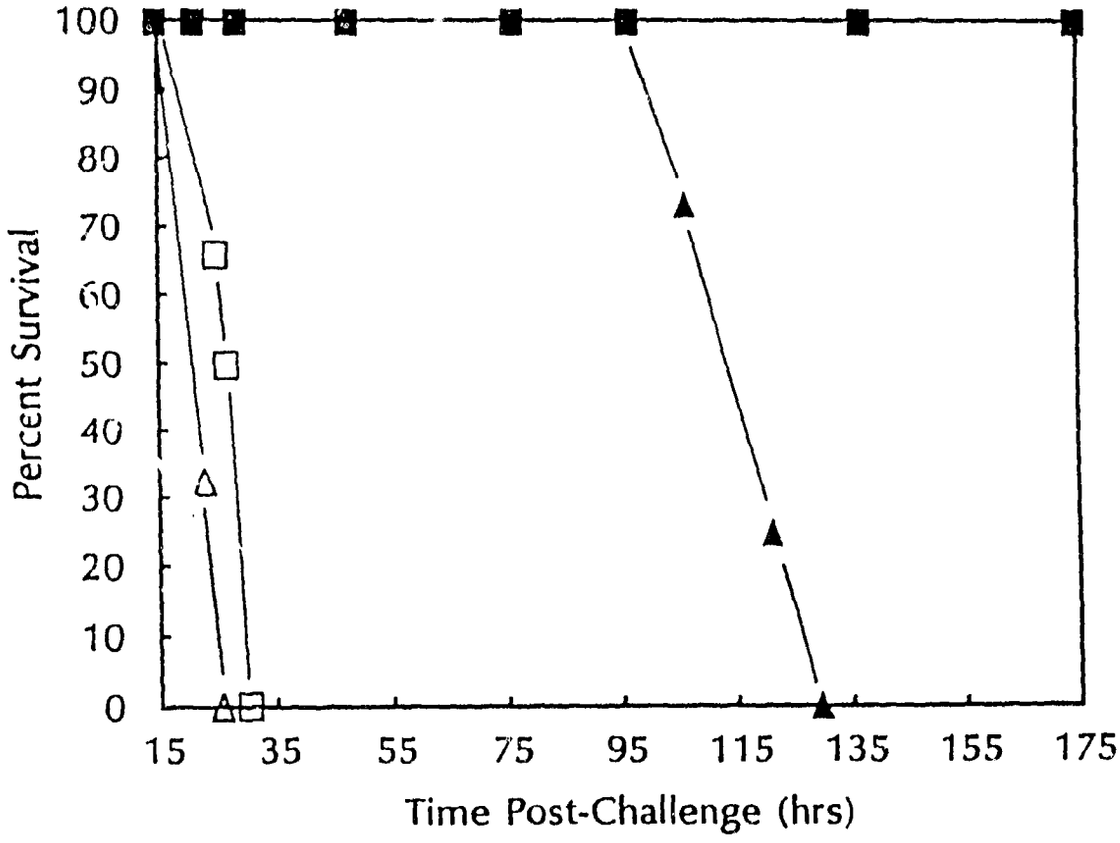


Fig. 3. Protection against ricin *in vivo* toxicity by infusion of BG11-G2 anti-ricin mAb before and after challenge with ricin. See legend of Figure 2.

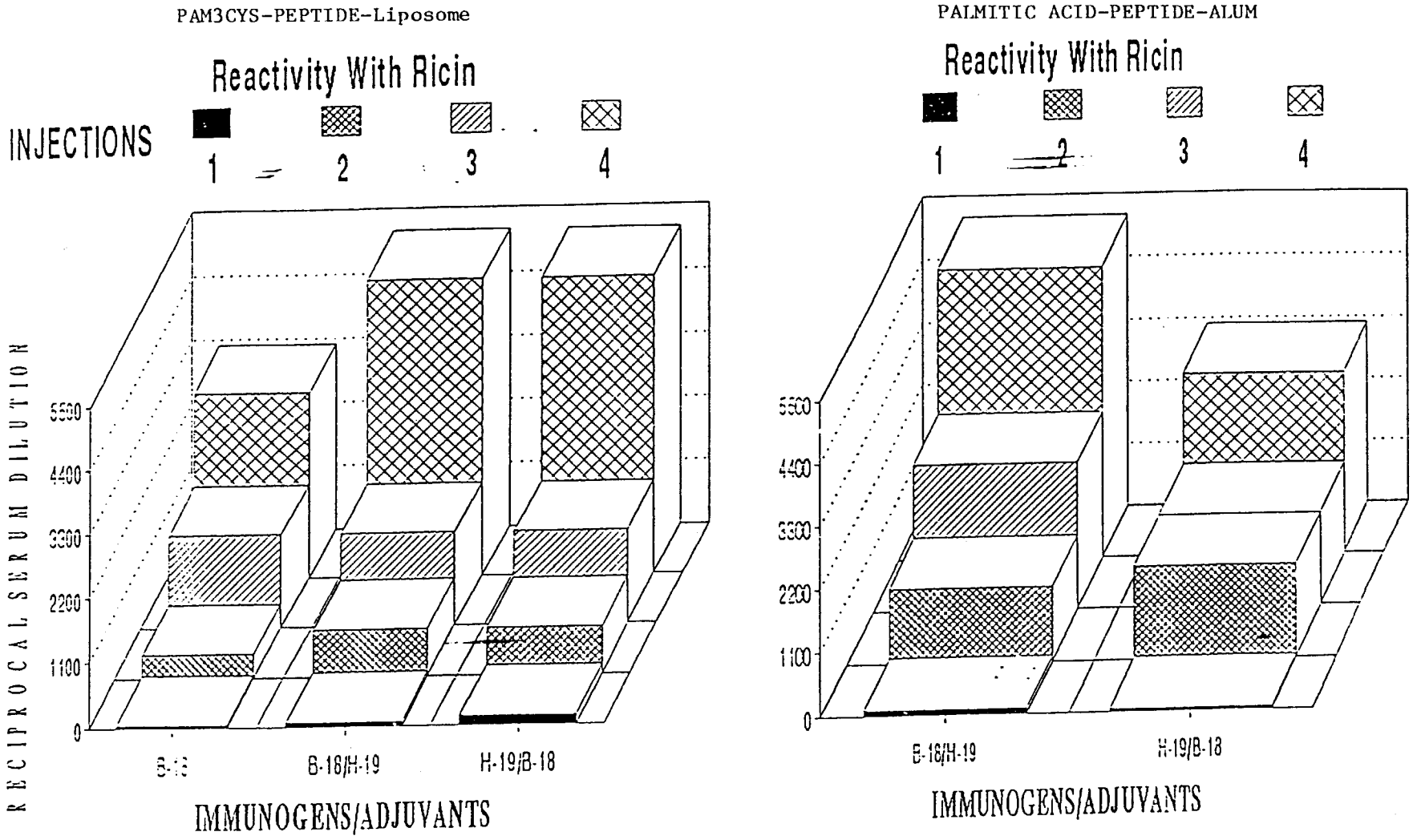

PAM3CYS-PEPTIDE-Liposome
Reactivity With Ricin
INJECTIONS
1
2
3
4
RECIPROCAL SERUM DILUTION
5500
4400
3300
2200
1100
0
B-18/H-19
H-19/B-18
IMMUNOGENS/ADJUVANTS
PALMITIC ACID-PEPTIDE-ALUM
Reactivity With Ricin
1
2
3
4
5500
4400
3300
2200
1100
0
B-18/H-19
H-19/B-18
IMMUNOGENS/ADJUVANTS

Figure 4

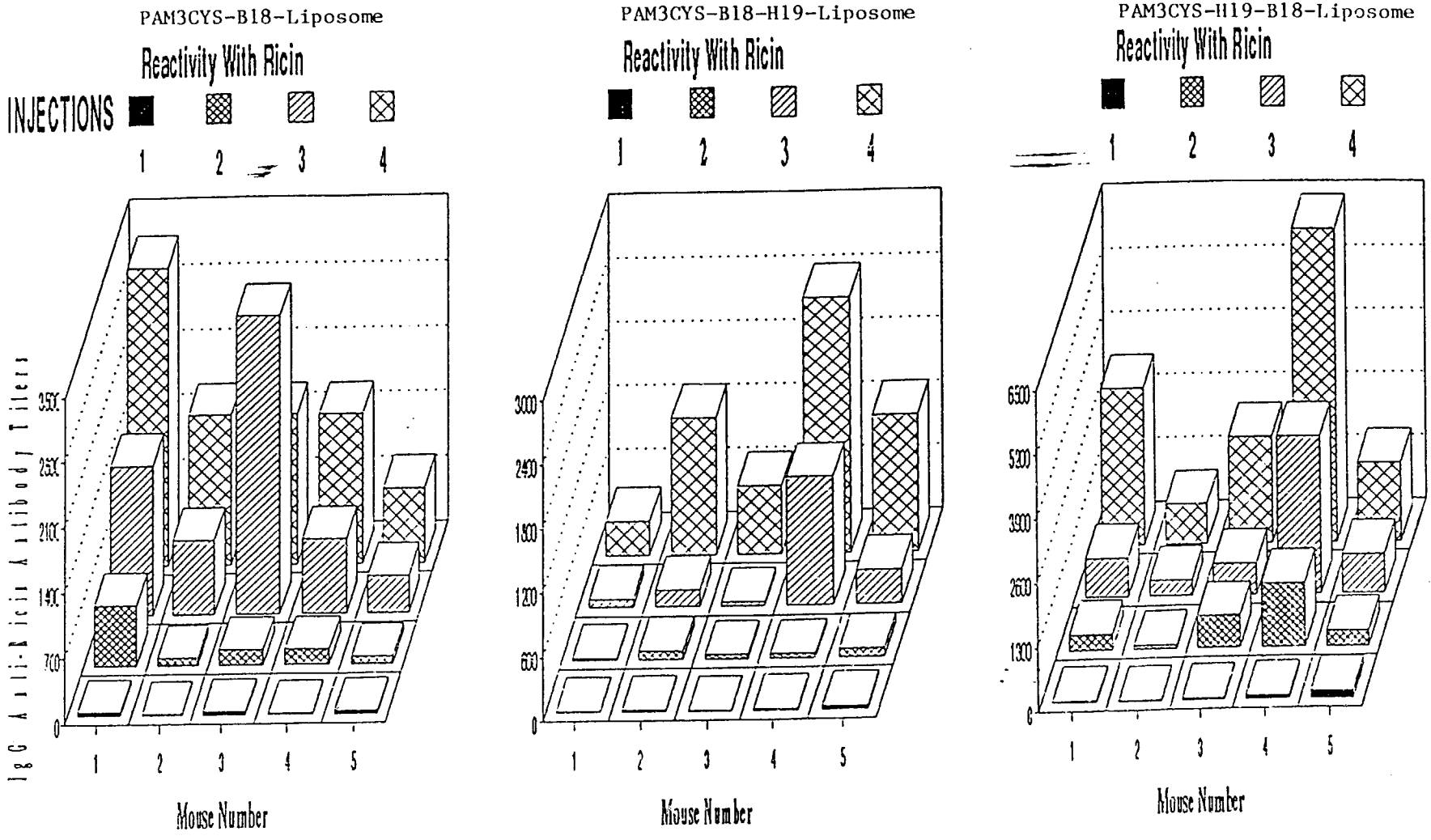
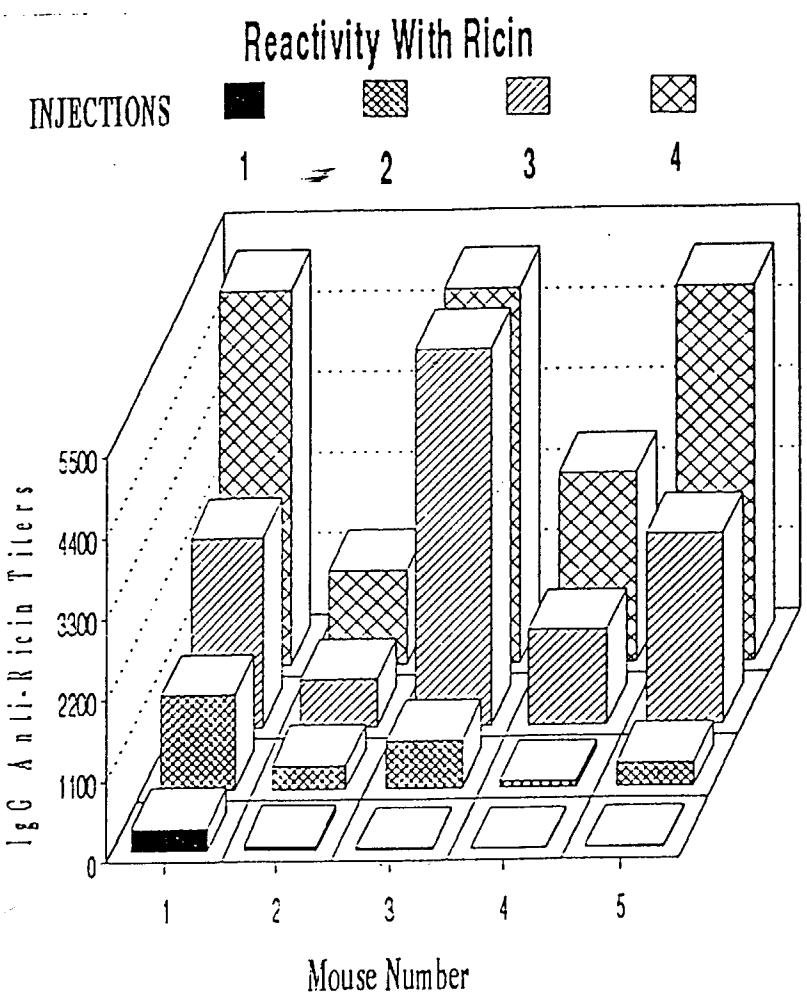

PAM3CYS-B18-Liposome
Reactivity With Ricin
INJECTIONS
1
2
3
4
IgG Anti-Ricin Antibody Titers
0
700
1400
2100
2800
3500
1
2
3
4
5
Mouse Number
PAM3CYS-B18-H19-Liposome
Reactivity With Ricin
1
2
3
4
0
600
1200
1800
2400
3000
1
2
3
4
5
Mouse Number
PAM3CYS-H19-B18-Liposome
Reactivity With Ricin
1
2
3
4
0
1300
2600
3900
5200
6500
1
2
3
4
5
Mouse Number

Figure 5

PALMITIC ACID-B18-H19



PALMITIC ACID-H19-B18

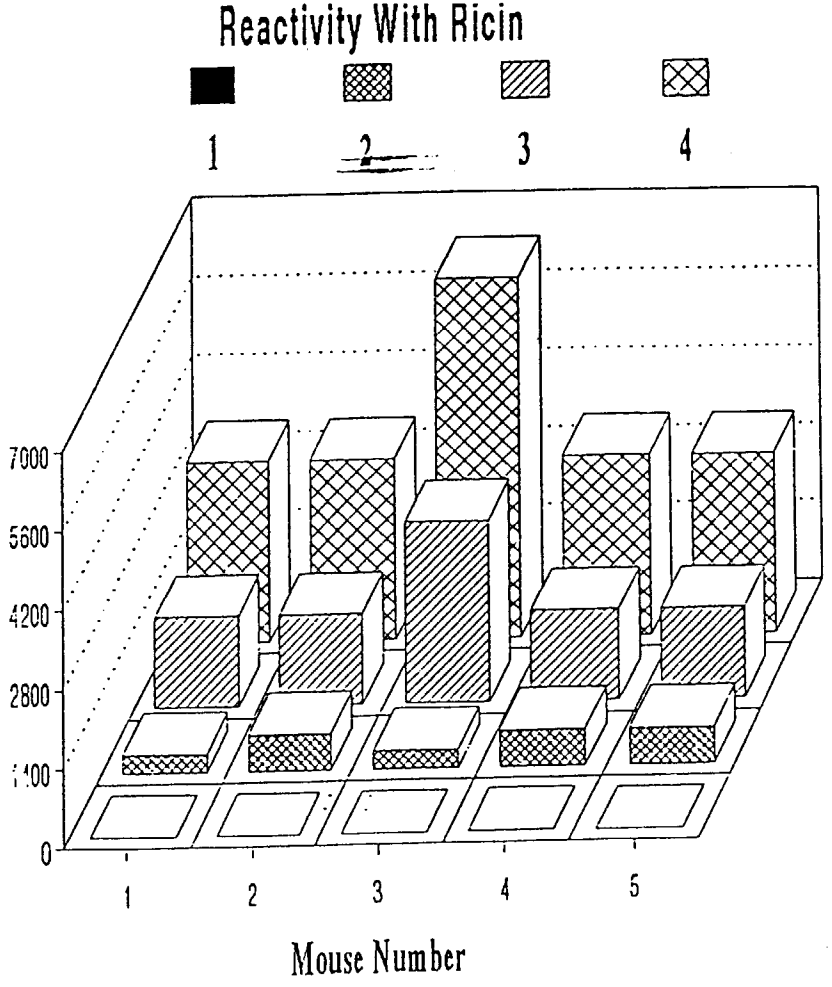
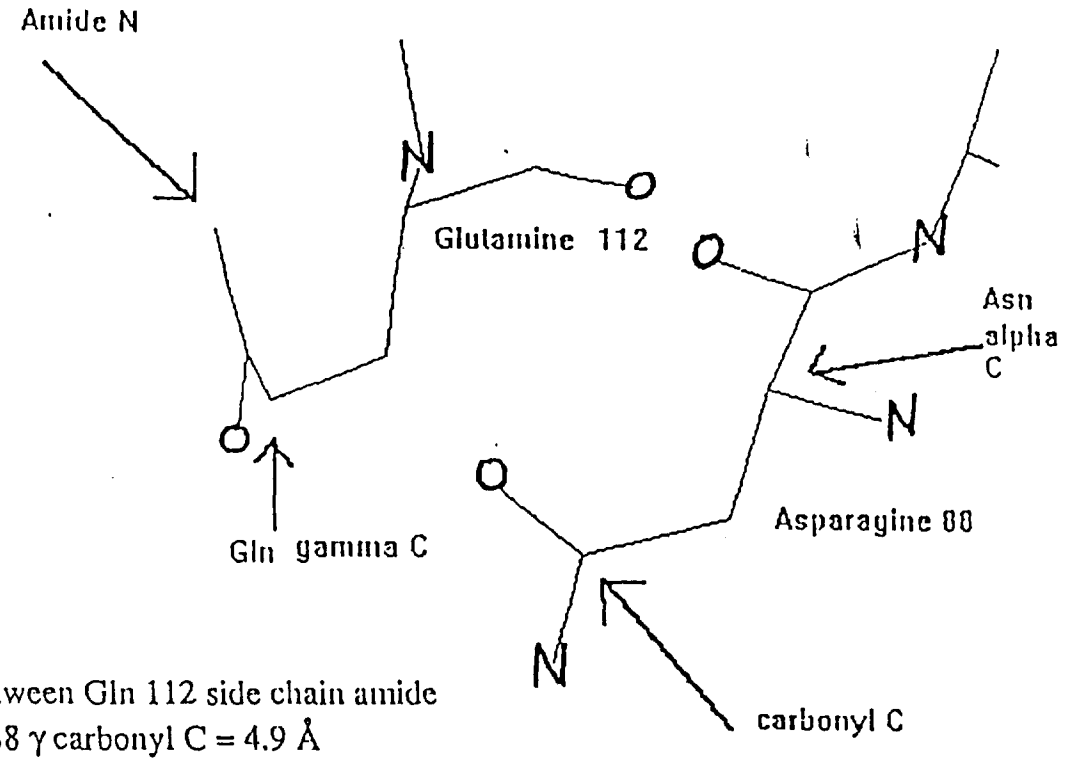


Figure 6



Distance between Gln 112 side chain amide N and Asn 88 γ carbonyl C = 4.9 Å

Distance between Asn 88 α C and Gln 112 γ C = 5.8 Å

Fig. 7 Expanded view of the stick model of the isolated glutamine 112 and asparagine 88 residues as contained within the Ricin A chain resolved at 2.5 Å. The distances between the atoms shown were determined using the ALCHEMY III program superimposed on the 2.5 Å structural model of Ricin created using the RASWIN program (Roger Sayle, Biocomputing Research Unit, Edinburgh, UK).

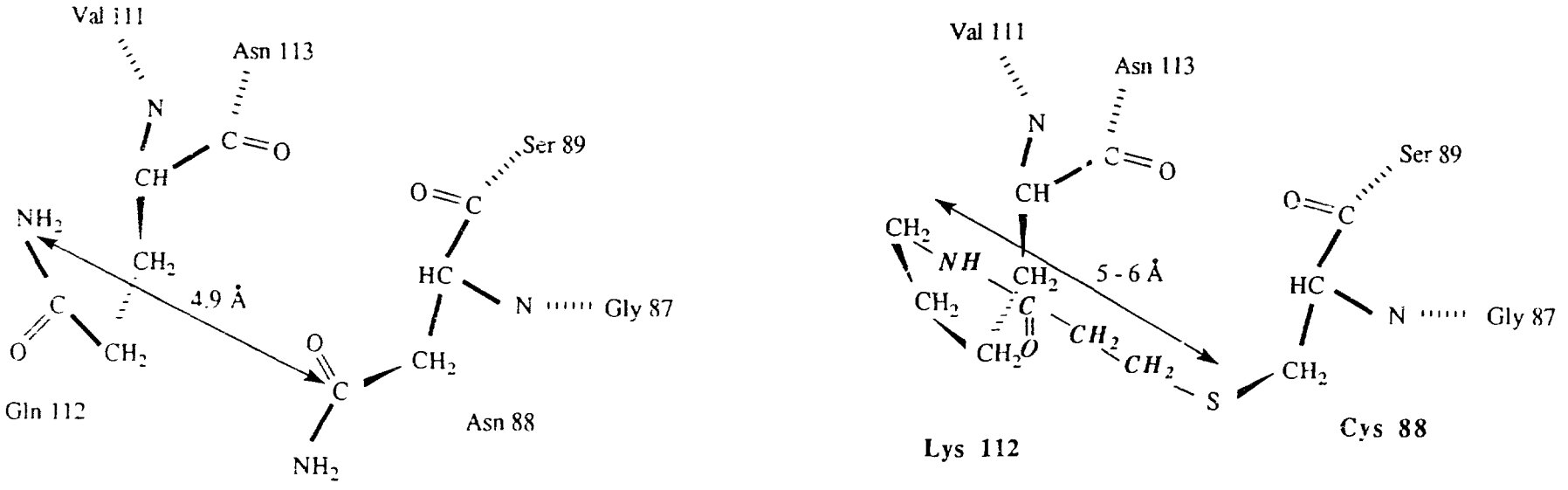
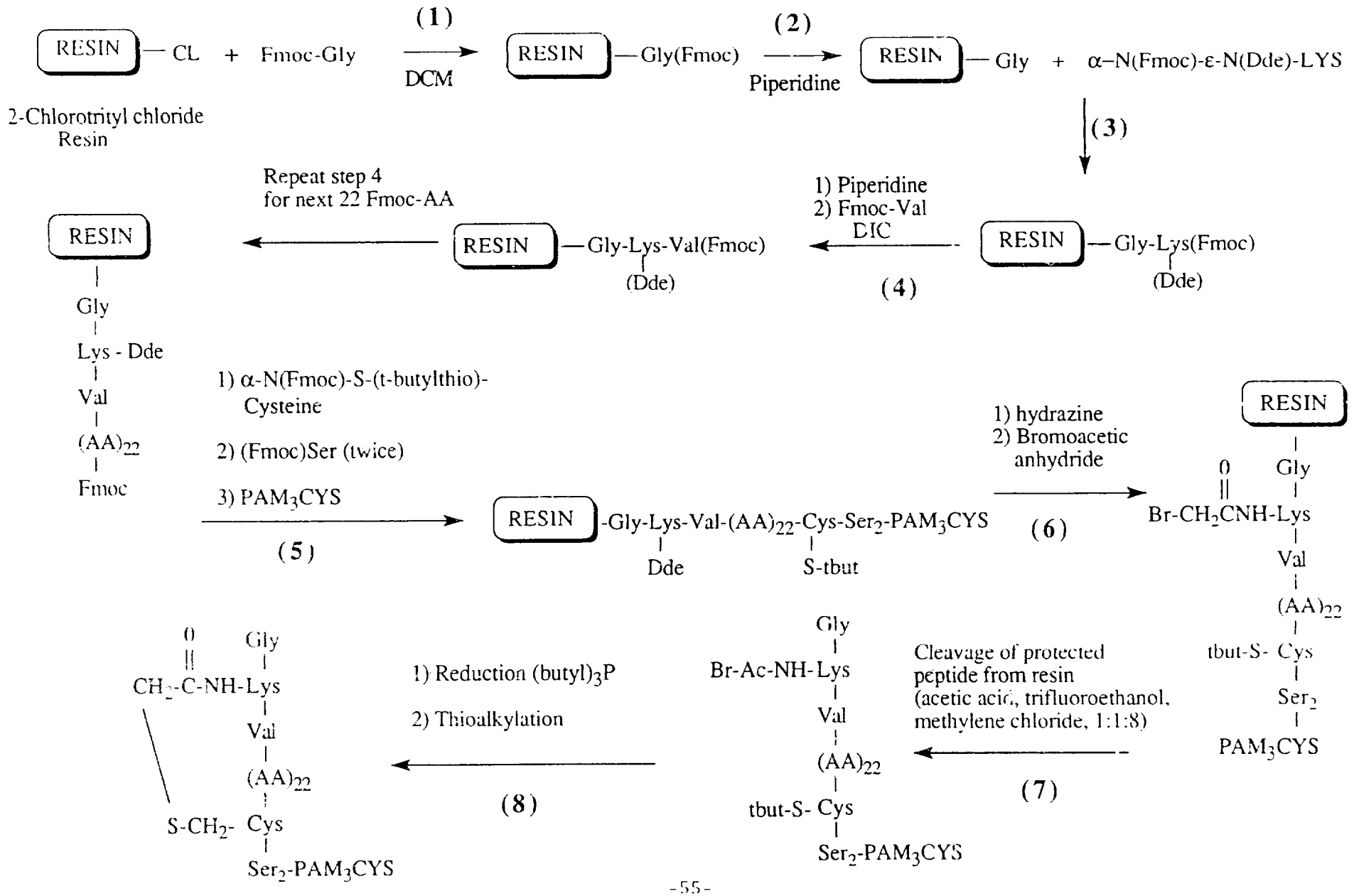


Fig. 8 Comparison of the spatial arrangement of the side chains of Asn 88 and Gln 112 as found in the 2.5 Å Ricin structure with one containing the Asn_{88}→Cys and Gln_{112}→Lys substitutions. If the Cys and Lys were oriented in a similar manner as the Asn and Gln of the native structure, the distance from the Cys sulfur atom to the Lys ε - CH_2 group would be about 5 - 6 Å as shown on the right. In the substituted peptide, this distance would be the approximate length of the four extra atoms (given in bold italics) introduced by the lysine (ε-amine) and propionyl crosslinker involved -***NH-C(O)-CH$_2$-CH$_2$***-.

SCHEME 1

SYNTHESIS OF CYCLIC RICIN A PEPTIDE 88-112



Appendix A

TOXICOLOGY AND APPLIED PHARMACOLOGY 108, 183–193 (1991)

CONTEMPORARY ISSUES IN TOXICOLOGY

Anti-idiotype-Based Vaccines against Biological Toxins[1]

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Anti-idiotype-Based Vaccines against Biological Toxins. CHANH, T. C., SIWAK, E. B., AND HEWETSON, J. F. (1991). *Toxicol. Appl. Pharmacol.* **108**, 183–193. Biological toxins produced by living organisms represent one of the major sources of contamination of stored grain and agricultural products, and other food sources. The majority of these biological toxins are highly lethal, nonproteinaceous low-molecular-weight chemical compounds which exert their potent toxicity through a variety of mechanisms. Because of their small size, they generally do not induce a significantly high affinity protective antibody response upon toxin exposure, even when conjugated to large protein carriers which enhance their immunogenicity. Moreover, the very toxic nature of biological toxins precludes their use as immunogens in the induction of protective immunity. To circumvent this difficulty, an attempt was made to develop antibody (anti-idiotype)-based vaccines against a protein synthesis inhibitor, the trichothecene mycotoxin T-2, and the sodium channel blockers tetrodotoxin and saxitoxin. Protective monoclonal antitoxin antibodies were first generated and then used to induce specific monoclonal anti-idiotype antibodies. Specific anti-idiotype antibodies were assessed for their ability to induce *in vivo* protective immunity against toxicity.

The term idiotype (Id),[2] or idiotypic determinant, originally proposed by Oudin and Michel (1963), represents the antigenic determinant or amino acid sequence associated with the variable (V) region of an antibody (Ab) molecule. Anti-Id (or Ab2) are specific anti-immunoglobulin (Ig) antibodies that can be used to define the Id (or Ab1) serologically. The V region of an antibody molecule represents the site at which interactions between antibody and antigen occur. The notion that the immune response to a nominal antigen (Ag) could be regulated by a series of Id–anti-Id interactions was first proposed by Jerne (1974). Jerne's network theory states that interactions between Id and anti-Id can either enhance or suppress the host's immune response to an Ag by feedback mechanisms. Within the network theory, it is also proposed that some anti-Id antibodies can mimic the three-dimensional structure of the nominal Ag. Antigen/antibody binding requires sufficient conformational complementarity between the Ag and the antibody molecule for the formation of interactive ionic forces necessary for this binding to occur. This conformational complementarity allows not only for antigen/antibody binding, but also for the interaction between anti-Id antibodies that rec-

[1] This research was supported by Contracts DAMD17-87-7005 and DAMD17-90-C-0051 from the U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD.

[2] Abbreviations used: Ab1, antiantigen; Ab2, antiantibody (anti-Id); b, biotin; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Id, idiotype; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; STX, saxitoxin; TDT, tetrodotoxin.

0041-008X/91 $3.00

ognize the three-dimensional conformation of the V region of the Ab1. Because of this structural complementarity, this class of anti-Id antibodies is recognized serologically as being similar to the nominal Ag and is said to be capable of "antigen mimicry."

Anti-Id antibodies can be classified into different categories based on their binding specificities (Bona and Kohler, 1984). Anti-Id antibodies recognizing determinants not associated with the antigen-binding site of the Ab1 (anti-Ag) and therefore not inhibited by the Ag are referred to as Ab2α. Antigen-inhibitable anti-Id antibodies can be divided into two classes. Anti-Id that are inhibited by antigen and that can display similar biological functions to that of the Ag (antigen mimicry) are designated Ab2β, or internal image anti-Id, whereas those that are inhibited by Ag but lack biological mimicry are referred to as Ab2γ.

ANTI-IDIOTYPES AS VACCINES

Internal image anti-Id or Ab2β have been proposed by Nisonoff and Lamoyi (1981) as potential antibody-based vaccines. Because of their biological mimicry of the Ag, it is theoretically feasible to substitute Ab2 anti-Id for the nominal Ag in the induction of a specific immune response. Indeed, a substantial body of evidence suggests the feasibility of using anti-Id antibodies as potential vaccines against a large number of infectious agents. The first report of an anti-Id vaccine for an infectious agent involved the *Trypanosoma* parasite (Sacks *et al.*, 1982). Successful anti-Id-based vaccines were also reported for hepatitis B (Kennedy *et al.*, 1983) and rabies (Reagan *et al.*, 1983) viral infections. A potential anti-Id vaccine for controlling infection by the human immunodeficiency virus (HIV-1) is currently being pursued (Chanh *et al.*, 1987; Dalgleish *et al.*, 1987). Infectious agents against which potential anti-Id vaccines have been reported are summarized in Table 1. Successful anti-Id vaccines have also been reported in some tumor systems (Herlyn *et al.*, 1986; Kennedy *et al.*, 1987), and in blocking pregnancy in mice (Wang *et al.*, 1989).

ADVANTAGES OF ANTI-IDIOTYPE VACCINES

There are several situations in which it is not only advantageous but also preferable to use the anti-Id-based vaccine approach (Table 2). The first and most obvious advantage would be in toxicology where, in most instances, the chemical nature and extreme toxicity of the compounds render conventional vaccine approaches, such as synthetic peptides and recombinant technologies or immunization with the attenuated form of the toxins, impractical. In these instances, the anti-Id-based approach may represent the only feasible alternative vaccine development strategy. This approach will be discussed in more detail below. A second advantage of an anti-Id approach would be in situations where sufficient amounts of purified immunogens are not readily available. A third potential advantage would be when the infectious agents exhibit nonprotective antigenic determinants which may be cross-reactive with the host's tissues. Anti-Id vaccines could induce an antibody response against a single selected protective antigenic determinant, thus avoiding the induction of antibody formation against the host's cross-reactive antigens which may lead to autoimmune complications. A fourth possible advantage is the ability of an anti-Id vaccine to serologically mimic a nonproteinaceous antigen, such as the case of some biological toxins and the polysaccharide capsule of some pathogenic microorganisms, and to induce protective immunity in animals that are genetically unresponsive immunologically to immunization with bacterial capsule until late in ontogeny (Stein and Soderstrom, 1984). The ability of anti-Id vaccines to prime the immune system for the selective production of protective antibodies directed against a specific antigenic determinant represents a fifth po-

tential advantage (Kennedy *et al.*, 1983; Reagan, 1985). This is particularly significant when dealing with toxin molecules containing specific active moieties. Neutralizing antibodies specific for the active sites on the toxin molecules can be induced by the use of anti-Id, without the generation of nonprotective antibodies. A sixth advantage would be with infectious agents exhibiting a relatively high degree of antigenic diversity while maintaining conserved protective epitopes (such as HIV-1 envelope glycoprotein tropism for the CD4 molecules expressed on the surface of human T-lymphocytes). Anti-Id vaccines can be designed to induce specific antibodies that prevent the binding of HIV-1 to these target cells (Chanh *et al.*, 1987; Dalgleish *et al.*, 1987).

STUDIES WITH THE TRICHOTHECENE MYCOTOXIN T-2

The trichothecene mycotoxins represent a large group of chemically related fungal metabolites produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma*, *Cephalosporium*, and *Stachybotrys*. The metabolites are nonproteinaceous compounds of approximate molecular weights of 300–500 kDa characterized by a basic tetracyclic sesquiterpene structure with a 12,13-epoxide group and a 9,10-oligenic bond (Bamberg and Strong, 1971). They represent a major source of contamination of stored grains and agricultural products (Ghosal *et al.*, 1978). The trichothecenes' wide range of biological activities include antibacterial, antifungal, and antiviral

TABLE 1

SUMMARY OF ANTI-IDIOTYPE-BASED VACCINES

Infectious agents	Diseases	Reference
Viral		
Hepatitis B virus	Serum hepatitis	Kennedy *et al.* (1983)
Rabies virus	Rabies	Reagan *et al.* (1983)
Reovirus	Encephalitis	Sharpe *et al.* (1984)
Sendai virus	Systemic infection	Ertl and Finberg (1984)
SV40	Murine tumors	Kennedy *et al.* (1985)
Poliovirus	Poliomyelitis	UytdeHaag and Osterhaus (1985)
Herpes simplex virus	Encephalitis	Gell and Moss (1985); Lathey *et al.* (1987)
Newcastle disease virus	Newcastle disease	Tanaka *et al.* (1986)
Feline leukemia virus	Feline immunodeficiency diseases	Uytdehaag *et al.* (1986)
Mouse mammary tumor virus	Mouse tumors	Raychaudhuri *et al.* (1987)
Human immunodeficiency virus	AIDS	Chanh *et al.* (1987); Dalgleish *et al.* (1987)
Influenza virus	Influenza	Mayer *et al.* (1987)
Cytomegalovirus	Latent disease	Keay *et al.* (1988)
Coxsackievirus	Myocarditis	Paque *et al.* (1988)
Bacterial		
Escherichia coli	Infantile diarrhea	Stein and Soderstrom (1984)
Group A streptococcus	Bacterial infection	Monafo *et al.* (1987)
Listeria monocytogenes	Meningitis	Kaufmann *et al.* (1985)
Mycobacterium leprae	Tuberculosis	Praputpittaya and Ivany (1987)
Streptococcus pneumoniae	Pneumonia	McNamara *et al.* (1987)
Parasitic		
Trypanosoma rhodesiense	Sleeping sickness	Sacks *et al.* (1982)
Trypanosoma cruzi	Chagas disease	Sacks *et al.* (1985)

Note. Adapted from Schick and Kennedy (1988).

TABLE 2

POTENTIAL ADVANTAGES OF ANTI-ID-BASED VACCINES

1. Antigen mimicry in toxicology where the toxic nature of the chemicals preclude their use as immunogens.
2. Inability to obtain sufficient immunogens (leprosy).
3. Induction of protective immunity against selected antigenic determinants while avoiding cross-reactive antibodies which may lead to autoimmunity.
4. Mimicry of nonproteinaceous immunogens (bacterial polysaccharides and biological toxins).
5. Priming of immune response for the production of desirable antibodies.
6. Infectious agents with high genomic diversity and conserved protective antigenic determinants (human immunodeficiency virus).

Note. Adapted from Kennedy and Chanh (1988).

properties, and all show some degree of animal toxicity. The trichothecene mycotoxin T-2 has been shown to be a potent inhibitor of protein synthesis (Cannon *et al.*, 1976; Schindler, 1974). T-2 exerts its toxicity by binding to the ribosomal subunit, resulting in the inhibition of peptidyl transferase function (Schindler, 1974). The LD50 values of T-2 administered gastrically in guinea pigs, rats, and mice are approximately 5.3, 7.0, and 9.6 mg/kg body wt, respectively (Fairhurst *et al.*, 1987).

Generation of a protective anti-T-2 monoclonal antibody. T-2 mycotoxin does not lend itself to conventional vaccine development approaches (synthetic peptide or recombinant technologies) since it is a small nonproteinaceous chemical compound. For immunization purposes, T-2 has been coupled to large protein carriers in order to induce a significant antitoxin antibody response. However, this does not represent a safe approach since conjugation of T-2 has been shown to result in unstable complexes with potential release of the toxin in its active form (Chu *et al.*, 1979).

To circumvent these problems, we have attempted to develop an anti-Id-based vaccine and to assess its potential in inducing protection against T-2 toxicity. The first step in developing an anti-Id antibody vaccine in this system is the generation of a specific anti-T-2 antibody (Ab1) that will confer protection against T-2 toxicity. BALB/C mice were immunized intraperitoneally with T-2 conjugated to poly-L-lysine (T-2-PLL) emulsified in complete Freund's adjuvant (CFA), followed 2 weeks later by T-2-PLL in incomplete Freund's adjuvant (Chanh *et al.*, 1989). Three more injections of T-2-PLL in phosphate-buffered saline (PBS) were given at 2-week intervals. The immune mouse spleen mononuclear cells were then fused with the mouse myeloma NS-1 cells to generate monoclonal antibodies (mAbs) as previously described (Gefter *et al.*, 1977). Resulting hybrids were assessed for their reactivity with T-2 mycotoxin in an enzyme-linked immunosorbent assay (ELISA) employing microtiter wells coated with T-2 conjugated to bovine serum albumin (BSA). A hybrid culture with the highest T-2 binding activity was selected and subcloned by limiting dilution, then injected into mice for ascites production, and the resulting mAb was purified by protein A–chromatography for further studies. The generated anti-T-2 mAb is an IgG_{1k} antibody and is designated HD11. HD11 mAb, but not a control IgG_{1k} mAb, specifically bound to T-2-BSA-coated wells in a dose-dependent fashion with a 50% binding end point at a concentration of approximately 0.2 μg/ml (Chanh *et al.*, 1989). There was no binding of HD11 to wells coated with two unrelated sodium channel blockers, saxitoxin (STX) and tetrodotoxin (TDT), conjugated to BSA (T. Chanh and J. Hewetson, manuscript submitted). The specificity of the binding was further assessed in inhibition ELISAs (Chanh *et al.*, 1989). As little as 6.0 ng/ml of T-2 inhibited approximately 30% of the binding of HD11 mAb to T-2-BSA, whereas 400 ng/ml of STX or TDT inhibited less than 10% of the binding (Fig. 1). The relative binding affinity constant of HD11 mAb was approximately 4.8×10^{-8} M as determined by IC50 logit analysis (Zettner, 1973). To assess the potential protection conferred by HD11 against T-2 cytotoxicity, an *in vitro* system measuring the incorporation of [^{3}H]leucine in the absence or presence of T-2 by the human epidermoid carcinoma cell

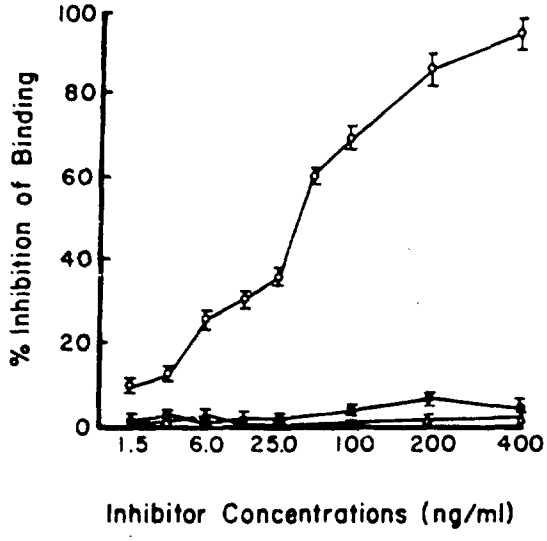

FIG. 1. Inhibition ELISA of HD11 mAb binding to T-2–BSA-coated wells. Each point represents the mean $OD_{410\ nm}$ ± SE of triplicate determinations. (○) T-2 mycotoxin; (●) saxitoxin; (▲) tetrodotoxin.

lines Hep-2 and KB was used. It was found that 10.0 and 16.0 ng/ml of T-2 inhibited approximately 50% of the radioactive leucine uptake by the KB and Hep-2 cell lines, respectively (Chanh *et al.*, 1989). When T-2, at a concentration that inhibited 50% of the leucine uptake, was incubated with HD11 mAb and added to the KB or Hep-2 cell monolayers, the cytotoxicity of T-2 was completely abolished (Table 3). Purified HD11 at a concentration of 1.0 μg/ml in the Hep-2 cultures almost completely neutralized T-2 cytotoxicity (3.6 and 62.3% inhibition of leucine incorporation with and without HD11, respectively). Likewise, addition of HD11 to T-2 in cultures of KB cells significantly enhanced the uptake of leucine. Incorporation of a control IgG_{1k} mAb with different specificity had no effect on T-2 cytotoxicity.

Generation of monoclonal anti-idiotypic antibody specific for HD11 anti-T-2. The purified and protective HD11 anti-T-2 mAb was conjugated to keyhole limpet hemocyanin (KLH) and used to immunize syngeneic BALB/C mice to generate monoclonal anti-Id antibodies (Chanh *et al.*, 1990). A syngeneic system was used to eliminate antiisotypic antibodies and to minimize antiallotypic antibodies. One week after the fourth immunization of HD11-KLH, spleen cells from the immune mice were fused with the NS-1 cell line. Since our strategy was to obtain T-2-inhibiting anti-Id, supernatant fluids from hybrid cultures were tested simultaneously for their ability to bind to HD11-coated wells and to inhibit the binding of HD11 to T-2–BSA-coated plates. Among approximately 800 hybrid culture supernatants tested, one was found that bound to HD11 and inhibited the latter from binding to T-2. This hybrid, termed

TABLE 3

PROTECTION AGAINST *IN VITRO* T-2 CYTOTOXICITY BY MONOCLONAL ANTIBODY HD11

	Hep-2 cells			KB cells		
Antibody (μg/ml)	No T-2	16 ng T-2/ml	%I	No T-2	10 ng T-2/ml	%I
None	403,412 ± 21,512[a]	151,007 ± 8,080	62.3[b]	298,387 ± 18,151	72,304 ± 6,819	75.8
HD11 (100)	463,724 ± 32,010	442,363 ± 25,905	4.6	309,240 ± 25,156	337,034 ± 28,816	0
HD11 (10)	436,929 ± 22,508	428,426 ± 21,115	1.9	299,334 ± 19,815	298,164 ± 19,150	0.4
HD11 (1.0)	419,008 ± 19,052	403,769 ± 18,456	3.6	278,158 ± 21,201	189,981 ± 12,100	31.7
HD11 (0.1)	429,342 ± 18,918	298,133 ± 15,615	30.5	301,619 ± 28,142	75,005 ± 7,015	75.1
HD11 (0.01)	415,905 ± 20,015	210,293 ± 16,518	49.4	311,479 ± 21,560	64,583 ± 8,001	79.2
Control IgG1(100)	389,641 ± 29,156	148,150 ± 10,108	61.9	286,168 ± 19,158	75,026 ± 7,105	73.8

[a] Mean of triplicate cpm ± SE.

[b] Mean percentage inhibition of [^{3}H]leucine uptake.

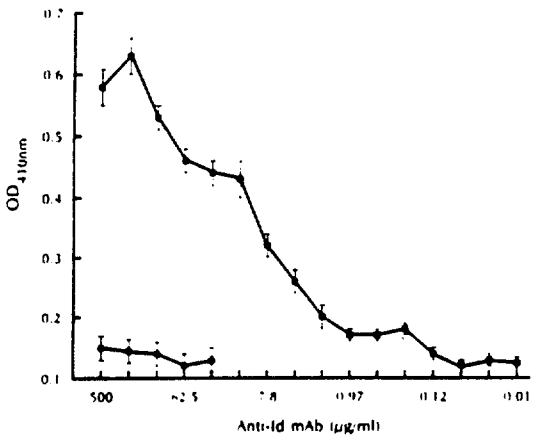

FIG. 2. Binding of DE8 anti-Id mAb to HD11-coated wells. DE8 mAb (■) and control anti-Id (●). Each point represents the mean ± SE of triplicate determinations.

DE8, was of the IgG_{2bk} isotype. The specificity of the binding of DE8 anti-Id to HD11 anti-T-2 was tested in a "sandwich" binding ELISA in which various concentrations of purified DE8 or of a control anti-Id mAb specific for an anti-hepatitis B surface antigen (gift from Dr. R. Kennedy, Southwest Foundation for Biomedical Research, San Antonio, TX) were added to HD11-coated wells (Chanh *et al.*, 1990). Binding reactivity was detected by addition of HD11 conjugated to biotin (HD11-b), followed by avidin–horseradish peroxidase (A-HRP) and the substrate 2,2-azino-di-3-ethyl benzthiazoline-6-sulfonate (ABTS). Figure 2 shows that the binding of DE8 to HD11 was dose-dependent with a 50% end point at a DE8 concentration of approximately 7.8 μg/ml. No significant reactivity was observed with the control anti-Id at a concentration of 0.5 mg/ml. In similar assays, HD11 was specific in its binding to DE8, whereas normal mouse Ig or control IgG1 mAb did not bind to DE8. These results suggest that DE8 mAb recognizes an Id determinant expressed by HD11 anti-T-2 antibody.

Characterization of the Id determinant recognized by DE8 anti-Id. To determine whether this recognition occurs at the T-2-binding site of HD11, an ELISA was performed in which various concentrations of DE8 mAb were incubated with a constant concentration of HD11-b prior to addition to T-2-BSA-coated wells (Fig. 3). The results showed that 3.6 μg/ml of DE8 was able to inhibit approximately 50% of the binding of HD11 to T-2, whereas the control monoclonal anti-Id antibody was unable to significantly affect the binding. To assess whether the HD11 Id determinant recognized by DE8 anti-Id is Ag-inhibitable, various concentrations of free T-2 mycotoxin were used to inhibit the binding of HD11-b to DE8-coated wells (Fig. 4A) and of DE8-b to HD11-coated wells (Fig. 4B). T-2 mycotoxin efficiently inhibited DE8/HD11 interactions, whereas STX had no significant effect on the binding. We also tested DE8 anti-Id for its ability to neutralize the protective effect of HD11 on the T-2-induced cytotoxicity. Various concentrations of DE8 or control anti-Id mAb were incubated with a protective concentration (100 μg/ml) of HD11 followed by the addition of a concentration of T-2 (16.0 ng/ml) predetermined to inhibit approximately 50% of leucine uptake by the Hep-2 cells (Fig. 5). As anticipated, HD11 at 100 μg/ml completely protected against T-2 toxicity (58,156 ± 3214 and 138,747 ± 9341 cpm without and with HD11, respectively, compared to 140,660 ± 11,101 cpm uptake by cultures without T-2). DE8 at concentrations of 50 μg/ml and higher significantly abolished the protective effect of HD11, indicated by a comparable uptake of radioactive leucine by

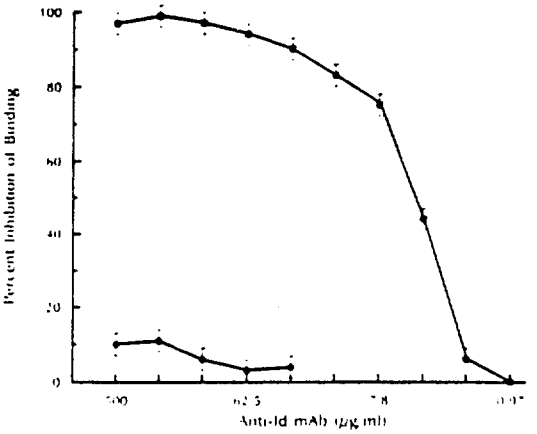

FIG. 3. Inhibition of HD11-b binding to T-2–BSA-coated wells by DE8 anti-Id. See legend to Fig. 2.

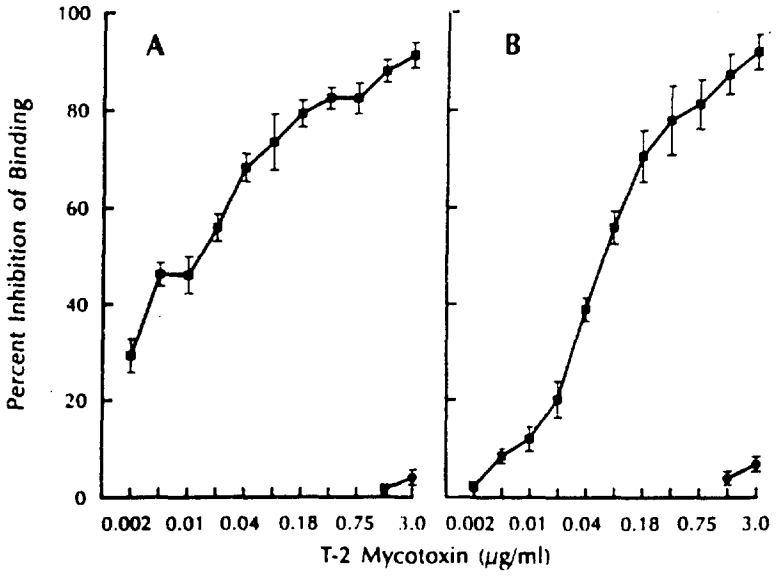

FIG. 4. Inhibition of HD11/DE8 interactions by T-2 mycotoxin. Inhibition of HD11-b binding to DE8-coated wells (A), and of DE8-b to HD11-coated wells (B) by T-2 (■) or by saxitoxin (●).

Hep-2 cells in the absence of HD11 (58,156 ± 3214 cpm) and in the presence of HD11 and 50 μg/ml of DE8 (69,991 ± 5173 cpm). An inversely proportional relationship was noted between the leucine uptake and the concentration of DE8. The control anti-Id mAb had little effect. Thus, it appears that the binding of DE8 to HD11 inhibited the latter from binding to T-2 and neutralizing its toxicity. These results strongly suggest that the Id determinant on HD11 recognized by DE8 anti-Id is associated with the T-2-binding site and that the HD11/DE8 interaction is inhibited by T-2 mycotoxin.

In vivo induction of a protective anti-T-2 immune response by DE8 anti-Id immunization. To test whether DE8 can mimic T-2 in inducing an *in vivo* anti-T-2 antibody response in a syngeneic system, four groups of BALB/C mice were immunized intraperitoneally with T-2-OVA, DE8, DE8-KLH, or a control anti-Id-KLH. After three immunizations, their sera were tested for T-2 binding to T-2–BSA-coated wells (Fig. 6). Immunization with the antigen (T-2-OVA) or with DE8-KLH induced a significant and comparable anti-T-2 antibody response. Administration of unconjugated DE8 resulted in a much lower level of anti-T-2 antibody response, which is consistent with previous results indicating the requirement of conjugating anti-Id to heterologous protein carriers in the induction of a significant syngeneic immune response specific for the nominal Ag (Ward *et al.*, 1987). Sera of mice immunized with the control anti-Id showed background reactivity.

The anti-T-2 antibody responses induced by DE8 or DE8-KLH were characterized by the presence of HD11 Id-positive antibodies. Sera

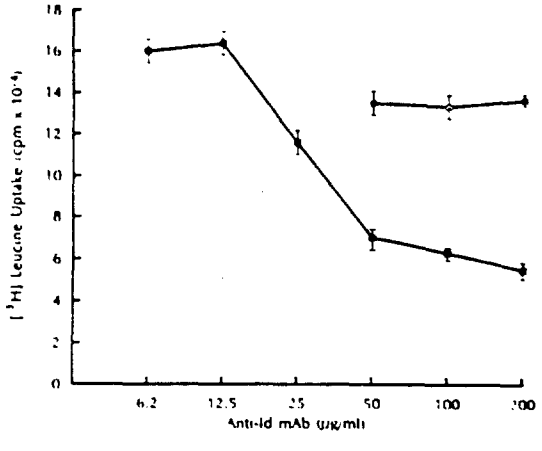

FIG. 5. DE8 anti-Id abolishes the protective effect of HD11 against T-2 cytotoxicity. DE8 anti-Id (■) and control anti-Id (●). The mean cpm ± SD of cultures with and without T-2 were 58,156 ± 3124 and 140,660 ± 11,101 cpm, respectively. The mean cpm ± SD of cultures with T-2 and 100 μg/ml of HD11 was 138,747 ± 9341 cpm.

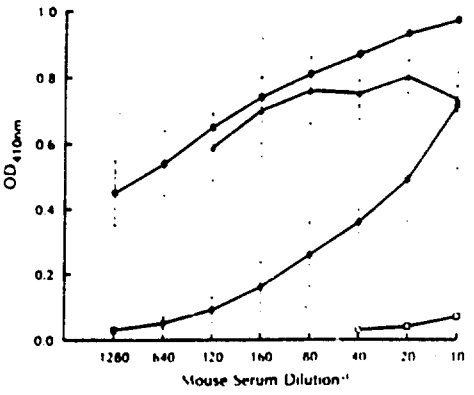

FIG. 6. *In vivo* induction of anti-T-2 antibodies by DE8 immunization. Mice were immunized with DE8-KLH (■), DE8 (●), T-2-OVA (▲), or control anti-Id (○). Each point represents the mean ± SD of five mice.

from mice immunized with DE8 or DE8-KLH inhibited the binding of DE8 to HD11, suggesting the presence of HD11-like anti-T-2 antibodies (Chanh *et al.*, 1990). Thus, immunization with DE8 appeared to specifically induce activation and clonal expansion of progenitor lymphocytes expressing surface HD11 Id-positive receptors, presumably through the binding of the anti-Id to these receptors followed by subsequent cell proliferation and antibody production. Administration of T-2-OVA did not result in detectable HD11 Id-positive antibodies although the overall anti-T-2 antibody response was comparable to that obtained with DE8-KLH immunization (Fig. 6). It is noteworthy that the immune response to a particular Ag can be modulated by anti-Id to consist of a specific Id determinant, in this case HD11 Id, which is associated with protection against T-2 toxicity. HD11 anti-T-2 mAb is the only protective antibody among approximately 50 other mAb with T-2 binding activity generated in our laboratory.

To determine whether the anti-T-2 antibody response induced by anti-Id immunization could protect against T-2 toxicity, various dilutions of sera from the four groups of immune mice were mixed with T-2 at 16 ng/ml and assayed for [^{3}H]leucine uptake on Hep-2 cells (Fig. 7). Incubation of DE8- and DE8-KLH-immune mouse sera with T-2 significantly reduced the T-2-induced cytotoxicity on Hep-2 cells at all dilutions tested (1:20 to 1:640). It is noteworthy that the T-2 neutralizing activity of sera from mice immunized with T-2-OVA was not as pronounced as that of DE8- or DE8-KLH-immune mice. Incubation of T-2 with T-2-OVA immune sera resulted in approximately 25 to 35% inhibition of radioactive leucine uptake, whereas incubation with DE8- or DE8-KLH-immune sera resulted in leucine uptake almost comparable to cultures without T-2 (59.3%). Sera from mice immunized with the control anti-Id had no effect. It is noteworthy that DE8 immunization, which modulated the anti-T-2 antibody response toward the expression of HD11-positive Id (see above) which is associated with protection, resulted in a superior protective immunity against T-2 toxicity than immunization with the nominal Ag (T-2-OVA). Encouraged by the results of the *in vitro* Hep-2 assay system, we assessed the ability of DE8-KLH to induce an anti-T-2 antibody response that would protect mice against a lethal challenge with T-2. Groups of five BALB/C mice each were immunized with four injections of DE8-KLH or control anti-Id mAb. One week after the last immunization, the mice were challenged intradermally

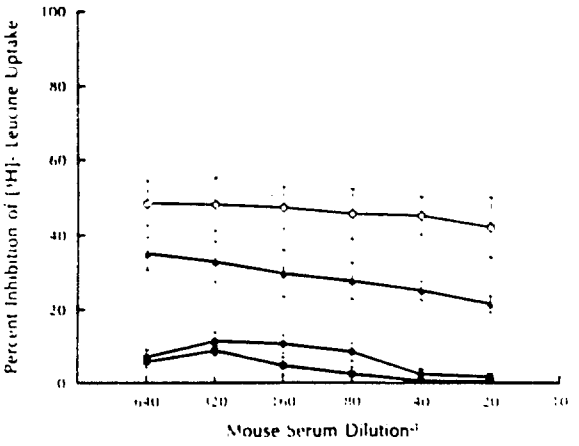

FIG. 7. *In vivo* induction of a protective anti-T-2 antibody response by DE8 immunization. See legend to Fig. 6. The mean cpm ± SD of Hep-2 cultures with and without T-2 were 43,135 ± 3891 cpm and 93,541 ± 9165 cpm, respectively (53.9% inhibition of uptake).

with various doses of T-2 mycotoxin. The results are presented in Table 4. It was previously determined that the LD50 of T-2 in naive mice challenged intradermally is approximately 5.0 mg/kg body weight. Mice actively immunized with DE8-KLH were protected against T-2 challenge. Although 1/5 mice in the group that received 10 mg/kg of T-2 succumbed to T-2 toxicity, all the remaining DE8-KLH-treated mice, even the group that received 20 mg/kg of T-2 (four times the LD50), survived. Mice treated with the control anti-Id antibody were not protected against T-2 toxicity. This represents to our knowledge the first demonstration of anti-Id-induced protective immunity against a low-molecular-weight biological toxin.

TABLE 4

ADMINISTRATION OF DE8 ANTI-IDIOTYPE PROTECTS MICE AGAINST T-2 TOXICITY *IN VIVO*

Mice immunized with	T-2 mycotoxin (μg/kg)	Survival ratio[a]
Control anti-Id	5.0	3/5
	10.0	4/5
	15.0	5/5
	20.0	5/5
DE8 anti-Id	5.0	0/4
	10.0	1/5
	15.0	0/5
	20.0	0/5

[a] Number of dead mice/total number of mice tested. T-2 mycotoxin was administered intradermally.

STUDIES WITH THE SODIUM CHANNEL BLOCKERS

The sodium channel blockers have generated a considerable amount of interest because of their ability to block sodium ion transport (Evans, 1972). Sodium current in nerve and muscle cells can be blocked with as little as 1–10 nM concentration of TDT (Colquhoun *et al.*, 1972). Death occurs through a rapidly progressive weakening of voluntary muscles, including respiratory muscles, resulting from the interruption of neuromuscular transmission in motor neurons (Cheymol *et al.*, 1962) and in muscle fiber membranes (Narahashi *et al.*, 1960). Our laboratory has initiated studies to assess the effectiveness of the anti-Id-based vaccine approach against saxitoxin and tetrodotoxin, two sodium channel blockers. STX and TDT, which are heterocyclic guanidinium compounds, are among the most potent poisons known, with LD50 values of approximately 10 μg intraperitoneally in mice (Bower *et al.*, 1981). Monoclonal Ab1 has been generated against STX (Huot *et al.*, 1989a) and TDT (Huot *et al.*, 1989b). Two mAbs (S1A5 IgM_k and S3E.2 IgG_{1k} mAbs) were isolated from fusions of spleen cells from BALB/C mice immunized with STX-KLH. They are specific for STX and do not bind to TDT, despite their relatively similar chemical structures (Huot *et al.*, 1989a). Although they both possess relatively low K_a values (10^6 M^{-1}), they were able to displace the binding of [^{3}H]STX to rat brain membranes, and to partially protect against STX-induced reduction of peripheral nerve action potential in rat tibial nerve (Huot *et al.*, 1989a). Likewise two monoclonal anti-TDT antibodies (both of the IgG_{1k} isotype) that protected against the TDT-induced reduction of nerve action potential (Huot *et al.*, 1989b) were generated. Syngeneic monoclonal anti-Id antibodies have been derived from these mAbs and are being assessed for their potential to induce *in vivo* protective immunity against the sodium channel blockers STX and TDT.

CONCLUDING REMARKS

We have presented experimental evidence to suggest the feasibility of the anti-Id-based vaccine approach in providing protection against the *in vivo* toxicity of the trichothecene mycotoxin T-2. Theoretically, it should be possible to design effective anti-Id vaccines against all toxic substances provided that antitoxin antibodies could first be generated that bind and neutralize circulating toxins before

they reach lethal levels in the blood stream. However, there are instances where this approach may not be feasible. One of the drawbacks of this approach results from an inherent property of antigen/antibody binding. Antigen/antibody interactions are primarily mediated by noncovalent ionic forces with binding affinity constants ranging between 10^{-6} and 10^{-10} M. This property imposes a limit on antibody-based protection against toxic substances possessing higher binding affinity constants for their target site(s). A case in point is represented by the organophosphorus compound soman which interacts covalently with its receptor site, acetylcholine esterase. Although specific monoclonal anti-soman antibodies have been generated, they only partially protected mice against soman toxicity, presumably due to their inefficiency in competing with the esterase for binding to soman (T. Chanh and J. Sadoff, unpublished results).

To our knowledge, the results presented in this review represent the first demonstration of an anti-Id-based vaccine capable of inducing protective immunity against a nonproteinaceous, low-molecular-weight biological toxin (T-2 mycotoxin). Efforts to develop successful anti-Id vaccines against STX and TDT toxicity are under way. These results suggest the possibility of "antigen mimicry" by anti-Id antibodies of biological toxins against which conventional vaccine development approaches cannot be applied because of their chemical nature, small size, and extreme *in vivo* toxicity. Moreover, the natural immune response to a particular toxin may comprise a majority of nonprotective antibodies directed against the nontoxic moieties of the toxin. Anti-Id vaccines with the potential ability to specifically induce the production of antitoxin antibodies expressing Id determinants associated with protection represent a definite advantage in vaccine development in toxicology. Thus, an anti-Id-based vaccine represents a viable and safe approach. Moreover, this vaccine strategy may possibly be the only alternative in providing protection against some of these biological toxins.

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Appendix B

RESEARCH

Anti-idiotype vaccines in toxicology

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Summary. The majority of naturally occurring biological and chemical toxins are highly lethal, nonproteinaceous, low molecular weight substances which exert their toxicity through a variety of mechanisms. Their relative small size and extreme in vivo toxicity have hampered the development of protective vaccines. We have investigated the feasibility of anti-idiotype-based vaccines which utilize antibodies for inducing a systemic and protective immunity against the in vivo toxicity of some of these toxic substances. A murine IgG_1 monoclonal anti-T-2 mycotoxin antibody protective against mycotoxin toxicity was generated. This antibody was used to produce a second generation monoclonal anti-idiotype antibody which was capable of serologically mimicking the tertiary conformation of the nominal antigen, i.e., T-2 mycotoxin. Administration of the monoclonal anti-idiotype antibody to mice induced a circulating and protective antibody response against the in vitro and in vivo toxicity of T-2 mycotoxin. Antibody-based vaccines may represent the only safe and effective strategy for the design of protective vaccines against small nonproteinaceous toxic compounds whose extreme toxicity prevents their use as safe immunogens. The potential of antibody-based vaccines for producing protective immunity against low molecular weight chemical and biological toxins is discussed.

Key words: Anti-idiotype antibody vaccines – T-2 mycotoxin – Saxitoxin – Tetrodotoxin – Ricin

Introduction

The trichothecene mycotoxins represent a chemically related group of fungal metabolites characterized by a basic tetracyclic sesquiterpene structure with a 12,13-epoxide moiety and a 9,10-oleginic bond [3]. This group of biological toxins, the most potent of which is the mycotoxin T-2, represents the major cause of a variety of fungal mycotoxicoses in humans and animals. The trichothecene mycotoxin T-2 is a potent inhibitor of mammalian protein synthesis, affecting the initiation step on polyribosomes and the elongation and/or termination steps [7, 51]. There is, at present, no effective prophylaxis against the in vivo toxicity of the trichothecene mycotoxins. T-2 mycotoxin is a low molecular weight compound (approximate formula weight 466.5), thus requiring conjugation to large protein carriers to render it immunogenic. However, currently available conjugation methods usually result in unstable T-2/protein carrier complexes with leakage of toxic free T-2. Such T-2/carrier complexes have been shown to be toxic when administered to animals, and therefore cannot serve as safe immunogens for the induction of protective immunity [12, 21].

Other chemical and biological toxins of interest include the sodium channel blockers, saxitoxin (STX) and tetrodotoxin (TDT), and the protein synthesis inhibitor ricin. Their chemical structures and attempts at generating anti-idiotype (Id)-based vaccines against their toxicity will be discussed below.

There are three main types of antigenic determinants associated with an immunoglobulin (Ig) or antibody molecule. The first is represented by isotypes or isotypic determinants. Isotypes define the class and subclass differences that are common to all Igs of a given species (i.e., γ, μ, α, ε, δ for the Ig heavy chain, and κ and λ for the Ig light chain). The second type of antigenic determinants found on Ig molecules is the allotypes or allotypic determinants. Allotypes are defined as intraspecies polymorphisms found on Ig molecules. Allotypes can be associated with either or both the constant or variable (V) regions of Ig molecules. The third type of antigenic determinants associated with Ig molecules are termed idiotypes (Ids) or Id determinants. Id determinants encompass antigenic determinants or amino acid sequences associated solely within the binding or V region of the antibody molecule [39]. Anti-Id, also termed Ab2 for the second antibody, can be generated by immunization with

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the Id-bearing antibodies, termed Ab1, and have been used to serologically define the Id. The binding of antibody to antigen requires sufficient conformational complementarity between the antigen and antibody molecules for the establishment of ionic forces necessary for this interaction to occur. This conformational complementarity necessary for antigen/antibody binding also allows for the interaction between anti-Id antibodies which recognize the three-dimensional conformation of the Id determinant located within the V region of the Ab1. As a result of this structural complementarity, this type of anti-Id antibodies are recognized serologically as being similar in structure to the nominal antigen, and are said to be capable of "antigenic mimicry".

Anti-Id vaccines

Anti-Id antibodies have been classified according to their binding specificities [5]. Those anti-Id antibodies specific for antigenic determinants not associated with the antigen-binding site of the Ab1 (antiantigen), and therefore not inhibited in their binding to Ab1 by the antigen, have been referred to as Ab2α. Anti-Id antibodies whose binding to Ab1 can be inhibited by the nominal antigen have been divided into two classes. Those Ab2 whose binding is inhibited by antigen but lack antigen mimicry are called Ab2γ, whereas those which are inhibited by antigen and which display similar serological properties to those of the antigen are designated Ab2β. As a result of this biological mimicry of the antigen, Ab2β anti-Id could theoretically substitute for the nominal antigen in the induction of a specific antibody response. In fact, Ab2β or internal image anti-Id antibodies have been proposed as potentially useful antibody-based vaccines [35]. Furthermore, within the last 10 years, a large number of studies have demonstrated the feasibility of the use of anti-Id antibodies as vaccines in a variety of biological systems. Most of these studies have focussed on Ab2β with serological mimicry to a variety of antigens, however, it has been demonstrated that Ab2α can also induce an antigen-specific immune response without subsequent antigen exposure [50, 61].

Anti-Id vaccines inducing protective immunity against infectious agents were first reported for *Trypanosoma rhodesiense* and *T. cruzi* [48, 49]. A number of studies have reported the use of anti-Id vaccines in viral infections. These include hepatitis B virus [26, 27], rabies virus [46], reovirus [17], mouse mammary tumor virus [42–45], Sendai virus [14], and human immunodeficiency virus-1 [2, 9, 61] infections. Experimental anti-Id vaccines have also been used successfully in some tumor systems [20, 28, 29, 42–45] and, interestingly, in blocking pregnancy in mice [56]. Anti-Id antibodies have also been used to identifiy specific cellular receptors involved in viral recognition and binding. Among these are the cellular receptors for reovirus [17, 59], Epstein-Barr virus [4], polyomavirus [31], and murine leukemogenic retrovirus [1]. A summary of major studies of anti-Id vaccines in viral, bacterial, and parasitic infections is given in Table 1.

Table 1. Summary of the major experimental anti-idiotype vaccines

Infectious agents	Diseases	References
Viral		
Hepatitis B virus	Serum hepatitis	26
Rabies virus	Rabies	46
Reovirus	Encephalitis	17, 59
Sendai virus	Systemic infection	14
Simian virus	Murine tumors	28
Poliovirus	Poliomyelitis	54
Herpes simplex virus	Encephalitis	18, 27
Newcastle disease virus	Newcastle disease	53
Feline leukemia virus	Feline immunodeficiency	55
Mouse mammary tumor virus	Mouse tumors	42–45
Human immunodeficiency virus	Acquired immunodeficiency syndrome	2, 9, 61
Influenza virus	Influenza	32
Cytomegalovirus	Latent disease	25
Coxsackievirus	Myocarditis	40
Bacterial		
Escherichia coli	Infantile diarrhea	52
Group A streptococcus	Bacterial infection	33
Listeria monocytogenes	Meningitis	24
Mycobacterium tuberculosis	Tuberculosis	41
Streptococcus pneumoniae	Pneumonia	57
Parasitic		
Trypanosoma rhodesiense	Sleeping sickness	48
T. cruzi	Chagas' disease	49

Anti-Id vaccine against T-2 mycotoxin toxicity

The trichothecene mycotoxins represent a major source of contamination of stored grains and agricultural products [19]. They exhibit a wide range of biological activities which include antifungal, antibacterial, and antiviral properties, and animal toxicity. T-2 mycotoxin, possibly the most toxic of the trichothecenes, exhibits a gastric LD_{50} (dose that kills 50% of the challenged animals) of approximately 5.3 mg/kg, 7.0 mg/kg, and 9.6 mg/kg in guinea pigs, rats, and mice, respectively [16]. T-2 mycotoxin is a low molecular weight (466.4 Da), nonproteinaceous toxin which renders the development of effective conventional vaccines (using such approaches as recombinant and synthetic peptide technology) difficult.

Protective murine monoclonal anti-T-2 antibody

To circumvent this difficulty, we have generated monoclonal (mAb) Ab1 anti-T-2 antibodies from BALB/C mice immunized with T-2 mycotoxin conjugated to poly L-lysine (PLL). The general scheme for the anti-Id approach in inducing protective immunity against toxic substances is presented in Fig. 1. An IgG_1 anti-T-2 mAb, designated HD11, was generated from fusions of spleen cells derived from T-2-PLL-immune mice with murine NS-1 myeloma cells [10]. Protein A-purified HD11 anti-T-2 mAb is specific for T-2 mycotoxin, and not for unre-

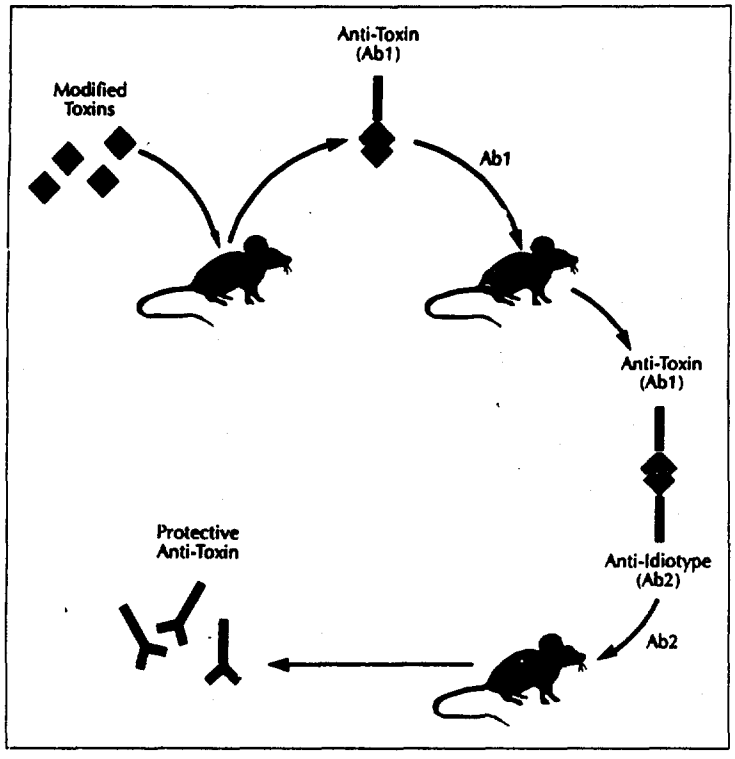

Fig. 1. Schematic representation of the anti-idiotype (Id)-based approach for the generation of protective immunity against toxins

lated toxins, and has a relative binding affinity constant of approximately 5.0×10^{-8} M, determined by IC_{50} (50% inhibitory concentration) logit analysis as described by Zettner [60]. To assess the potential protection of HD11 against T-2 cytotoxicity, an in vitro system using the T-2-sensitive human epidermoid carcinoma cell lines Hep-2 or KB was used. Incubation of HD11 mAb with T-2 mycotoxin completely inhibited the in vitro cytotoxicity of T-2 [10].

Generation and characterization of anti-Id mAbs specific for HD11

Purified HD11 mAb, derived from BALB/C mice, was conjugated to keyhole-limpet hemocyanin (KLH) and used to immunize BLAB/C mice for the generation of syngeneic anti-Id mAbs [11]. This syngeneic system was used to minimize anti-isotype and antiallotype reactivity. Since our screening strategy was to select for hybrids secreting anti-Id mAbs specific for the T-2-binding site(s) on HD11 Ab1, supernatant culture fluids resulting from the fusions were screened simultaneously for their ability to bind to HD11-coated microtiter wells and to inhibit the binding of HD11 mAb to wells coated with T-2 conjugated to bovine serum albumin (BSA). Among approximately 800 hybrid culture supernatants screened, 1 IgG_{2b} anti-Id mAb, termed DE8, was isolated that recognized an Id determinant associated with the HD11 mAb binding site. Further characterization using purified DE8 anti-Id mAb showed that: (1) DE8 inhibits the binding of HD11 to T-2-BSA-coated wells (Fig. 2); (2) free T-2 mycotoxin is capable of inhibiting both the binding of

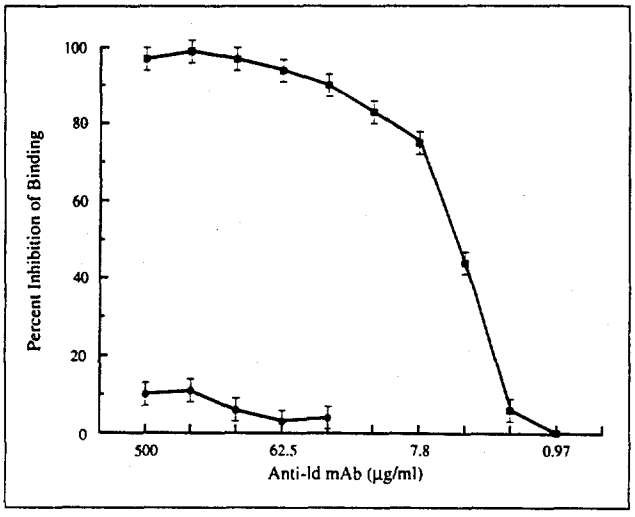

Fig. 2. DE8 monoclonal (*mAb*) anti-Id antibody (■) inhibits the binding of HD11 anti-T-2 to T-2-bovine serum albumin-coated microliter wells. ●, Control anti-Id mAb

HD11 to DE8-coated wells and that of DE8 to HD11-coated wells (Fig. 3); (3) DE8 mAb negates the protective effects of HD11 against the in vitro cytotoxicity of T-2, presumably by inhibiting HD11 mAb from binding to and neutralizing T-2 [11]. These results suggest that HD11/DE8 interactions can be inhibited by T-2 mycotoxin, and that DE8 anti-Id mAb recognizes the T-2-binding site(s) on HD11 mAb.

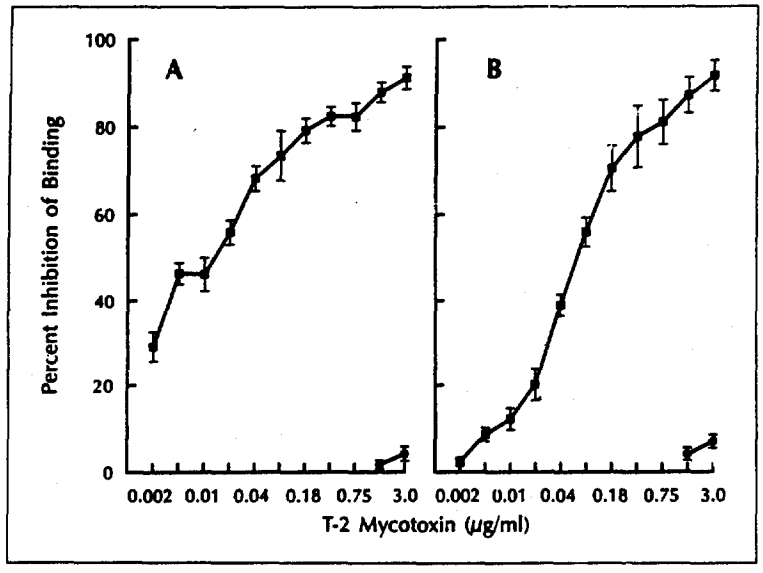

Fig. 3. Inhibition of HD11 Ab1/DE8 Ab2 interactions by T-2 mycotoxin (■) or by saxitoxin (●). **A** Inhibition of HD11 mAb binding to DE8; **B** inhibition of DE8 mAB binding to HD11. The mean percentage inhibition was determined as: {[optical density (OD) of binding in the absence of toxin]−[OD of binding in the presence of toxin]/[OD of binding in the absence of toxin]} × 100

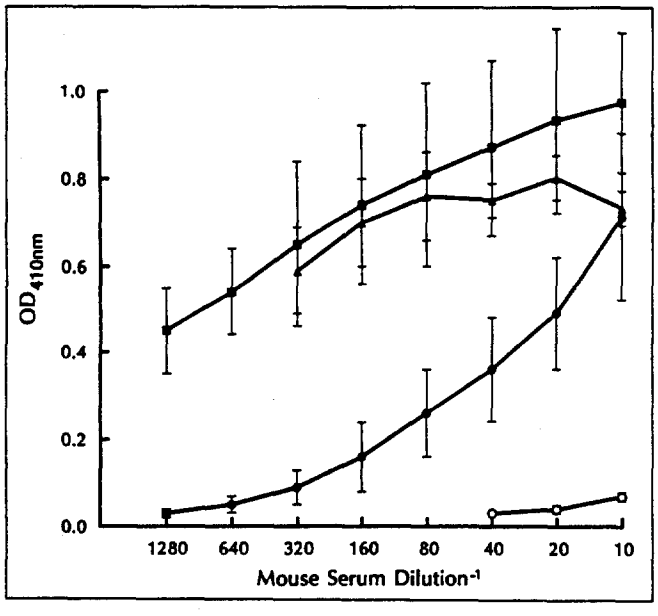

Fig. 4. In vivo induction of anti-T-2 antibody responses by vaccination with DE8 mAb. BALB/C mice were immunized with DE8 *Keyhole-impet hemocynin* (■), unconjugated DE8 (●), T-2-ovalbumin (▲), or control anti-Id mAb (○). Each value represents the mean ± SD of five mice

Generation of a protective anti-T-2 immune response by vaccination with DE8 anti-Id

To assess whether DE8 mAb could serologically mimic the nominal antigen (i.e., T-2) in inducing in vivo a protective anti-T-2 antibody response in a syngeneic system, four groups of BALB/C mice were immunized intraperitoneally with T-2 conjugated to ovalbumin (OVA), DE8 mAb, DE8 mAb-KLH, or a control anti-Id mAb-KLH. Two weeks after the third immunization, the mice were tested for binding to T-2 using microtiter wells coated with T-2-BSA (Fig. 4). Immunization with T-2-OVA or with DE8-KLH generated a significant and comparable anti-T-2 antibody response. In contrast, immunization with unconjugated DE8 anti-Id resulted in a lower anti-T-2 immune response. This is consistent with previous observations that conjugation of the anti-Id antibody to heterologous protein carriers is necessary for the induction of a significant antibody response against the nominal antigen in a syngeneic system [57]. Another noteworthy observation is the ability of sera from mice immunized with anti-Id (DE8-KLH or DE8) to inhibit the binding of DE8 to HD11, suggesting the presence of HD11-Id$^+$ anti-T-2 antibodies in the generated anti-T-2 immune response [11]. Administration of T-2-OVA did not induce detectable HD11-Id$^+$ anti-T-2 antibodies, although the overall anti-T-2 response was comparable to that obtained with DE8-KLH immunization, and was higher than that obtained with unconjugated DE8 (Fig. 5). Thus, anti-Id vaccination can direct the antibody response against a particular antigen towards a specific Id determinant, in this case HD11 Id which is associated with protection against T-2 toxicity. HD11 anti-T-2 mAb represents the only protective mAb among the 50 or so mAbs with T-2 specificity generated in our laboratory [8]. Immune sera from mice immunized with T-2-OVA, or with the anti-Id mAb DE8, or DE8-KLH were shown to be capable of protecting against the cytotoxicity of T-2 mycotoxin in the Hep-2 cell assays [11]. It is noteworthy that the T-2-neutralizing activity of DE8- and DE8-KLH-immune sera is higher than that of T-2-OVA immune sera. This may be accounted for by the DE8 anti-Id-induced positive modulation of HD11 Id$^+$ anti-T-2 associated with protection.

These results argue for possible mimicry of the tertiary conformational structure of T-2 mycotoxin by DE8 anti-Id mAb. The in vitro results discussed above led us to assess the ability of DE8-KLH to induce in vivo an anti-T-2 antibody response that would protect mice against a lethal challenge with T-2 mycotoxin. Groups of five BALB/C mice each were given four injections of DE8 mAb-KLH or a control anti-Id mAb conjugated to KLH. One week after the fourth immunization, the mice

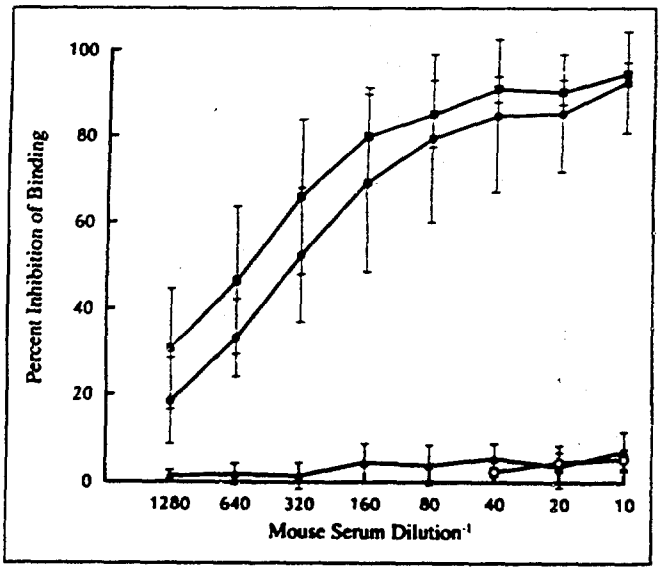

Fig. 5. In vivo vaccination with DE8 anti-Id mAb induces an HD11 Id^+ anti-T-2 antibody response (see Fig. 4). The mean percentage inhibition was assessed: [(OD of binding without sera)−(OD of binding with sera)/OD of binding without sera)] × 100

Table 2. In vivo induction of protective immunity by vaccination with anti-idiotype antibody

BALB/C mice vaccinated with	T-2 mycotoxin (mg/kg)	Survival ratio[a]
Control	5.0	3/5
Anti-idiotype	10.0	4/5
	15.0	5/5
	20.0	5/5
DE8	5.0	0/4
Anti-idiotype	10.0	1/5
	15.0	0/5
	20.0	0/5

[a] Number of dead mice/total number of mice challenged

were challenged subcutaneously with various doses of T-2 mycotoxin (Table 2). Mice actively immunized with DE8 anti-Id mAb conjugated to KLH were protected against an in vivo challenge with a lethal dose of T-2 mycotoxin. All the DE8-KLH-immune mice in the group receiving 20 mg/kg (4 times the LD_{50}) survived the challenge, although one of five mice receiving 10 mg/kg died. Immunization with the control anti-Id conjugated to KLH did not provide any protection. To our knowledge, this represents the first demonstration of in vivo anti-Id-induced protective immunity against a nonproteinaceous small molecular biology toxin.

Studies with other chemical and biological toxins

The sodium channel blockers STX and TDT

The sodium channel blocker STX and TDT have generated a considerable amount of interest in toxicology because of their ability to block sodium ion transport [15]. They represent some of the most poisonous substances known, being toxic at nanomolar concentrations in blocking sodium currents in nerve and muscle cells [13]. They are heterocyclic guanidinium compounds with LD_{50} values of approximately 10 μg in mice [6]. Our laboratory has generated two mAbs, namely S1A5 (IgM) and S3E.2 (IgG_2), specific for STX with relative K_a constants of approximately 10^{-6} M. Although their K_a values are relatively low, they are able to displace the binding of tritiated-[^{3}H]STX from rat brain membranes, and to protect against STX-induced reduction of peripheral nerve action potential in rat tibial nerve [22]. Similarly, two mAbs specific for TDT, both of the IgG_1 isotype with K_a values approaching 10^{-7} M, were isolated and shown to protect against TDT-induced reduction of nerve action potential [23]. The generation of syngeneic anti-Id mAbs and their potential as vaccines in inducing protective immunity are presently under investigation.

The protein synthesis inhibitor ricin

Ricin, a potent protein synthesis inhibitor, is produced by seeds of the castor plant *Ricinus communis*. Originally isolated by Stillmark in the 1880s, ricin is among the most studied and the most toxic chemicals known to man. A single ricin molecule in the cytosol is sufficient to cause cell death. Ricin is a disulfide-bonded heterodimer consisting of a 32-kDa A chain glycoprotein linked to a 32-kDa B chain glycoprotein [37]. The ricin B chain promotes binding of the ricin molecule to the cellular membrane through its binding specificity for complex galactosides [34]. This binding to cell surface receptors is believed to trigger the endocytic uptake of the whole ricin molecule. Once inside the cell, the ricin A chain dissociates from the B chain and quickly mediates the enzymatic attack on the cellular protein synthesis machinery at the level of the 60-S ribosomal subunit [38]. Although the exact mechanism of protein synthesis inhibition by ricin is not clearly understood, it is believed that ricin inactivates the 60-S ribosomal subunit catalytically without the requirement for cofactors. This has led to the notion that ricin may have endonuclease activity. Recent analysis of ricin-treated ribosomes has revealed that an adenine is missing from position A_{4324} although the phosphoribose backbone remains intact [36]. It is of interest that the adenine at position A_{4324} is located in a region of the ribosome which is highly conserved during speciation. It was further shown that ricin removes 1 adenine per ribosome subunit, suggesting that it may represent the only site of action of ricin toxicity.

We have recently generated an antiricin mAb, designated RG-2 (IgG_1), from a fusion between spleen cells from BALB/C mice immunized with a sublethal dose of whole ricin and the murine myeloma cell line Sp2/0. This mAb bound specifically to whole ricin- and ricin A chain-coated wells, but not to ricin B chain-coated wells, and protected against ricin cytotoxicity as assessed by [^{3}H]leucine uptake in an in vitro EL-4 cell assay (data not shown). The fine specificity of RG-2 and the generation

of anti-Id mAbs specific for RG-2 which could be utilized as vaccine for the in vivo induction of a protective antiricin antibody response are under investigation.

Concluding remarks

This review article presents experimental evidence to suggest the efficacy of the anti-Id-based vaccine strategy in generating protective immunity against the in vivo toxicity of the protein synthesis inhibitor T-2 mycotoxin. A similar approach is being investigated for another protein synthesis inhibitor, ricin, and the sodium channel blockers, STX and TDT. In theory, it should be feasible to design effective anti-Id vaccines against all toxic chemicals with the provision that sufficiently high-affinity binding antitoxin antibodies (Ab1) can be generated that can compete with the target sites for binding to and neutralization of circulating toxins before they reach lethal levels in the blood. The neutralizing Ab1 can then be used as immunogen to generate the proper type of anti-Id which mimics the tertiary conformation of the nominal antigen. The requirement of Ab1 possessing high-binding affinity constants represents one of the drawbacks of the anti-Id approach. Antigen/antibody interactions are primarily mediated by noncovalent ionic forces with relative binding affinity constants ranging approximately between 10^{-6} M and 10^{-9} M. This property imposes a limit on antibody-based protection against toxic substances with higher binding affinity constants for their target site(s) than those of antibody/toxin interactions. A case in point is seen with the organophosphorus compound, soman, more commonly known as nerve gas, which interacts covalently with its receptor site, acetylcholinesterase. Although specific monoclonal antisoman antibodies have been generated in our laboratory, they only partially protect against the in vivo toxicity of soman in mice. This is most likely accounted for by the inability of the antisoman mAbs to compete efficiently against the esterase for binding to soman (T. Chanh and J. Sadoff, unpublished observations).

Since its first proposal as a potential vaccine approach in 1981 [35], anti-Id vaccines have been described in a variety of experimental situations. There are several instances in which the anti-Id vaccine approach is not only advantageous but also preferable to conventional vaccine development strategy. The first and most obvious advantage would be in toxicology, as is discussed herein, where, in most instances, the chemical nature and extreme toxicity of the chemicals render conventional vaccine approaches impractical if not impossible. When dealing with toxic, nonproteinaceous low molecular weight toxins whose extreme toxicity precludes their use as immunogens, the only approach for the development of a safe and effective vaccine may be the use of anti-Ids. Moreover, as seen with the T-2 mycotoxin system, immunization of mice with T-2-OVA results in the generation of a good anti-T-2 antibody response in which only a minor proportion of the antibodies is associated with protection [8]. In contrast, DE8 anti-Id immunization also results in a good anti-T-2 antibody response, but one in which there is an increase in HD11 Id expression. Thus, immunization with DE8 anti-Id mAb appeared to induce a more protective anti-T-2 antibody response than did immunization with the antigen, T-2-OVA.

The anti-Id approach would also be useful where immunogens are difficult to purify in sufficient amounts, e.g., hepatitis B and C viruses which can not be propagated in large scale in vitro. Some infectious agents may also express nonprotective antigenic determinants which cross-react with the host's tissue antigens. Anti-Id vaccines could be designed to induce antibodies against a single selected protective determinant, thus avoiding the induction of antibodies against the host's tissues antigens which could lead to autoimmunity. A fourth possible advantage is represented by the ability of an anti-Id vaccine to serologically mimic polysaccharide capsule antigens of some pathogenic microorganisms [52, 58], and to induce protective immunity in animals that are genetically unresponsive immunologically to immunization with bacterial capsules until late ontogeny [52]. Finally, anti-Id may be useful in enhancing or priming the Id or antigen specificity of a given immune response by activating silent or suppressed clones of cells [32, 50].

The application of the anti-Id vaccine strategy in toxicology is still in its infancy. Attempts to modulate immune responses utilizing the anti-Id approach have been reported for bacterial toxins including streptococcal group A carbohydrate antigen [33], cholera toxin [30], the capsular polysaccharide antigen of *Neisseria meningitidis* [58], and staphylococcal enterotoxin [47].

The results presented herein represent the first demonstration of an anti-Id vaccine capable of generating protective immunity against a nonproteinaceous, low molecular weight biological toxin (T-2 mycotoxin). Our work suggests that anti-Id antibodies can serologically mimic the tertiary conformational structure of biological toxins against which conventional vaccine approaches cannot be applied because of their chemical nature, small size, and extreme in vivo toxicity. Furthermore, the antigen-induced immune response to a particular toxin may be directed primarily against nontoxic moieties of the toxin molecule, and therefore would not be highly protective. Anti-Id vaccines with the potential ability to specifically direct the antibody response toward the production of antibodies expressing an Id determinant associated with protection (or directed against the toxic structure of the molecule) represent a definite advance in vaccine development in toxicology. Thus, anti-Id vaccines, which could be purified to homogeneity without too much difficulty, represent not only a viable and safe approach, but maybe the only alternative vaccine development strategy against some biological toxins.

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Appendix C

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IMPHAR 00666

Polyclonal anti-idiotypes induce specific anti-saxitoxin antibody responses

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Abstract: Polyclonal BALB/C mouse and New Zealand White rabbit anti-idiotypic antibodies were raised by immunization with a protein G-purified burro anti-saxitoxin IgG antibody preparation. Following absorption of non-anti-idiotype reactivity, murine and rabbit IgG were purified by protein A chromatography and used to immunize BALB/C mice for the induction of anti-saxitoxin antibody responses. Unconjugated BALB/C anti-idiotypes did not induce significant anti-saxitoxin reactivity in BALB/C mice, even after repeated immunizations. However, BALB/C mice immunized with purified BALB/C anti-idiotypes conjugated to keyhole limpet hemocyanin, or with purified, unconjugated rabbit anti-idiotypes, as aluminum hydroxide precipitates, induced significant and specific anti-saxitoxin immune responses. Saxitoxin, a sodium channel blocker, can protect cells treated with veratridine and ouabain, whose respective actions are to open sodium channels and to block the activity of Na/K-ATPase. The anti-idiotype-induced anti-saxitoxin antibodies inhibited saxitoxin from protecting against cell death induced by veratridine and ouabain treatment. These and other published experimental results strengthen the concept of anti-idiotype-based vaccines in eliciting protective immunity against a variety of low molecular weight, nonproteinaceous biological and chemical toxins, whose extreme toxicity does not allow their use as safe immunogens.

Key words: Saxitoxin; Sodium channel blocker; Anti-idiotype; Vaccine; Protective immunity

Introduction

There exist three major categories of antigenic determinants within an immunoglobulin (Ig) or antibody molecule. Isotypic determinants represent the class and subclass differences common to all Igs of a given species, whereas allotypic determinants represent Ig intraspecies polymorphism. Isotypes are generally associated with the constant (C) region of the Ig molecule, whereas

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Abbreviations: Ab, antibody; ABTS, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonate); BSA, bovine serum albumin; C, constant; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Id, idiotype; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; NGS, normal goat serum; O.D., optical density; PBS, phosphate buffered saline; STX, saxitoxin; TDT, tetrodotoxin; V, variable.

allotypes can be found on both the C and variable (V) regions. The third type of antigenic determinant is represented by the idiotype (Id) determinant. The Id of an antibody molecule includes a collection of amino acid sequences, termed idiotopes, which reside solely within its binding or V region domain (Oudin and Michel, 1963). Anti-Id reagents, or anti-antibodies (Ab2), generated by immunization with the Id-bearing antibody (Ab1) have been used to define the Id serologically. The ability of anti-Id antibodies to modulate the in vivo immune responses against a number of antigenic systems has been documented (Bona, 1988). Perhaps the most interesting application of immune modulation by anti-Id antibodies is based on their ability to mimic the tertiary structure of the nominal antigen. This has led to the concept that 'internal image' anti-Id could be potentially useful as antibody-based vaccines (Nisonoff and Lamoyi, 1981). Indeed, the feasibility of this approach has been documented in a variety of viral, bacterial, and parasitic infections (Bona, 1988), and in cancer (Kennedy et al., 1987). Vaccination of mice with an anti-Id mAb raised against a protective mAb1 specific for the protein synthesis inhibitor trichothecene mycotoxin T-2 was recently shown by us to induce in the vaccinated animals a circulating and protective immune response against the in vitro and in vivo toxicity of T-2 mycotoxin (Chanh et al., 1990, 1992).

Saxitoxin (STX) is a potent neurotoxin with an approximate intraperitoneal LD_{50} (dose killing 50% of the injected animals) of 10 μg/kg in mice (Bower et al., 1981). It is a nonproteinaceous, heterocyclic guanidinium compound of approximate M_w of 300 Da, produced by dinoflagellates of the genus *Gonyaulax* during ocean blooms such as the red tide (Schantz et al., 1966). These organisms are ingested in large amounts by shellfish such as clams and mussels which concentrate STX, yet they themselves do not appear to be affected (Schantz, 1980). However, paralysis and death may occur in humans consuming the contaminated shellfish. The toxicity of this paralytic poison results from its ability to block sodium ion transport across the cell membrane (Evans, 1972). STX binds with an approximate dissociation constant of 5×10^{-9} M (Hille, 1968) to its cellular receptor, which is thought to reside on the external surface of the cell membranes rather than inside the sodium channel itself (Kao, 1968). This binding appears to be associated with a 260 kDa protein moiety as well as one or more smaller polypeptides (Tanaka et al., 1986).

To assess the feasibility of anti-Id based vaccine in the induction of protective immunity against STX toxicity, BALB/C mice and New Zealand White rabbits were immunized with protein G-purified burro anti-STX IgG, which had been previously demonstrated to reverse STX-induced cardiorespiratory failure in guinea pigs (Benton et al., 1991). The generated murine and rabbit anti-Id antibodies were purified and shown to induce a specific anti-STX antibody response in naive BALB/C mice. The anti-STX Ab3 containing sera were shown to negate the ability of STX to block the sodium channels of neuroblastoma cells treated with veratridine and ouabain. These results reinforce the potential effectiveness of anti-Id based vaccine in the induction of systemic and protective immune responses against biological and chemical toxins whose extreme toxicity prevents their use as safe immunogens.

Materials and Methods

Burro anti-saxitoxin IgG

The IgG fraction of burro anti-STX antibodies was purified by absorption and elution from protein G, and was generously provided by Dr. J.F. Hewetson (Pathophysiology Department, USAMRIID, Fort Detrick, Frederick, MD).

Generation of murine and rabbit anti-idiotype antisera

Female BALB/C mice (4–6 weeks of age) and female New Zealand White rabbits (4–6 months of age) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Upon arrival, both species of animals were quarantined for at

least 2 weeks before use. The burro anti-STX IgG preparation was precipitated with alum hydroxide as detailed elsewhere (Schick et al., 1989). Immunizations of the burro anti-STX IgG/alum in rabbits (50 μg/animal; intramuscular) and in mice (10 μg/animal; intraperitoneal) were done every 2 weeks, with bleedings obtained before each injection.

Absorption of polyclonal anti-idiotype antisera

To remove anti-isotype and anti-allotype antibodies, the polyclonal rabbit and mouse sera were repeatedly absorbed over columns of agarose conjugated to normal horse Ig (Sigma Chemical Co., St. Louis, MO). Normal horse Ig was used instead of normal burro Ig because it is more readily available and because of the close phylogenetic relationship between these two species.

Detection of anti-idiotype reactivity

To assess the anti-Id specificity of the absorbed sera, microtiter wells were coated with 0.05 ml of phosphate buffered saline (pH 7.2) containing 2.0 μg/ml of purified normal horse Ig (Sigma Chemical Co.) or purified burro-anti STX IgG overnight at 4 °C. The wells were washed with PBS/1% Tween-20 (wash buffer), and blocked with PBS/5% normal goat serum (NGS) for 1 h at 37 °C. The wells were washed with wash buffer, serial dilutions of the absorbed rabbit or mouse sera were added (0.05 ml) and the wells were incubated for 1 h at 37 °C. Pooled preimmune mouse and rabbit sera served as negative controls. After incubation, the wells were washed, and 0.05 ml of a proper dilution of goat anti-rabbit Ig (for detection of rabbit anti-Id) or anti-mouse Ig (for detection of mouse anti-Id) conjugated to horseradish peroxidase (HRP, Fisher Scientific, Orangeburg, NY) were added. The wells were incubated for an additional hour at 37 °C, followed by addition of the substrate 2, 2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonate, ABTS) in 0.3% 0.1 M citric acid buffer, pH 4.0 (Sigma Chemical Co.). The reaction was terminated by adding 0.1 ml of 5% SDS in PBS, and absorbance was determined at 410 nm in a Dynatech microplate reader. Determinations were done in triplicate.

Preparation of anti-Id for immunization

Absorbed rabbit or mouse anti-Id sera that reacted with burro anti-STX IgG, but not with normal horse Ig as assessed above, were further purified for BALB/C mouse immunization. Both absorbed rabbit and mouse anti-Id sera were absorbed to and eluted from protein A chromatography as described (Ey et al., 1978). The protein A-purified rabbit and mouse Ab2 were precipitated with aluminum hydroxide (Schick et al., 1989). Groups of BALB/C mice were immunized i.p. with 50 μg/mouse of purified rabbit or mouse Ab2 every other week for the generation of Ab3. Mice were bled before each immunization. The mouse Ab2 was also conjugated to KLH (Calbiochem, La Jolla, CA) as previously described for induction of Ab3 in another group of BALB/C mice (Schick et al., 1989).

Reactivity of purified mouse and rabbit anti-Id with mouse monoclonal anti-STX antibodies

To test for the ability of the purified mouse and rabbit Ab2 to bind to murine anti-STX mAbs, two previously generated anti-STX mAbs, namely S3E.2 (IgG_{1k}) and S1A5 (IgM_k), were used (Huot et al., 1989a). Briefly, purified S3E.2 or S1A5 anti-STX murine mAb1s were used to coat microtiter wells (3.0 μg/ml, 0.05 ml/well) overnight at 4 °C. The wells were washed and blocked as described above, followed by addition of serial dilutions (0.05 ml/well) of purified rabbit or mouse Ab2. The ELISA were performed as described above with the following changes. For the detection of rabbit Ab2 binding to mouse anti-STX, goat anti-rabbit Ig-HRP (Fisher Scientific) was used as the secondary antibody reagent. For the detection of mouse Ab2 binding, specific goat anti-mouse μ-chain-HRP (Fisher Scientific) was used for detecting the binding to S3E.2 mAb1 (IgG), whereas specific goat anti-mouse γ-chain-HRP (Fisher Scientific) was used for detecting the binding to S1A5 mAb (IgM).

Detection of anti-STX (Ab3) antibody response

Anti-STX antibody reactivity in sera of mice immunized with mouse or rabbit Ab2 was detected in an ELISA using STX conjugated to bovine serum albumin (STX-BSA) with formaldehyde as the coupling agent as previously described (Huot et al., 1989a). Goat anti-mouse Ig-, μ-chain-, and γ-chain-HRP were used to detect anti-STX antibodies of all isotypes, IgM isotype, and IgG isotype, respectively. Monoclonal S1A5 (IgM_k) and S3E.2 (IgG_{1k}) anti-STX previously generated by us (Huot et al., 1989a) were used as positive controls. Positive reactivity is defined as O.D. values of immune sera greater than 0.1 and greater than three times that of preimmune sera. Specificity analyses were done using binding ELISA with wells coated with an unrelated toxin (tetrodotoxin, TDT) conjugated to BSA (Huot et al., 1989b), and inhibition ELISA in which free STX was added as the inhibitor of binding to STX-BSA as previously described by us (Huot et al., 1989a).

Inhibition of sodium channel blocking by STX with anti-Id-induced anti-STX antibodies

An in vitro assay was employed to assess the ability of the murine anti-STX antibody responses, induced by rabbit and murine anti-Id, to abolish the protection provided by STX which blocks sodium channels opened up by treatment with veratridine and ouabain (Gallacher and Birkbeck, 1992). Mouse neuroblastoma cells (CCL131 obtained from the American Type Culture Collection, Rockville, MD) in RPMI 1640 (Cell Culture Laboratories, Cleveland, OH) supplemented with 25 mM Hepes, 200 IU/ml penicillin, 200 μg/ml streptomycin, and 10% FCS (Intergen, Purchase, NY) were plate in 96-well flat-bottom tissue culture plates (3×10^4 cells/well). Following overnight incubation at 37 °C, the culture medium was removed and replaced with fresh medium containing various concentration of ouabain and veratridine. We had predetermined that a combination of 0.5 mM ouabain and 0.25 mM veratridine kills approximately 50–70% of the CCL131 cells. It was also determined that STX at 30 nM provided approximately 50% protection against the ouabain veratridine mediated toxicity. To test for the ability of anti-STX Ab3 sera to negate the protection provided by STX in this system, mixtures of STX (30 nM) without or with serial dilutions of anti-STX Ab3 sera, or preimmune sera as negative controls, were added to CCL131 cell monolayers in the presence of 0.5 mM ouabain and 0.25 mM veratridine. The wells were incubated at 37 °C for 48 h, and cell viability was determined using the tetrazolium salt MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Chemical Co.) assay essentially as described (Mosmann, 1983). *t*-Distribution tests at 95% confidence level were used to determine the significance of the differences observed. Significant differences indicate that the anti-STX Ab3 did not negate the protective effect of STX in this system.

Results

Generation of murine and rabbit anti-Id antibodies

Following three immunizations with burro anti-STX IgG, the absorbed and protein A-purified murine and rabbit anti-Id antibodies showed significant binding against burro anti-STX IgG. Approximately 5.0 μg/ml of absorbed and purified murine anti-Id (Fig. 1) and 2.0 μg/ml of rabbit anti-Id (Fig. 2) exhibited about 50% binding to wells coated with burro anti-STX IgG, respectively. No significant ELISA reactivity was observed with either anti-Id preparations against purified normal horse Ig. Pooled preimmune rabbit and murine sera at 1:10 dilution did not react significantly with either burro anti-STX IgG, or with normal horse Ig (data not shown).

Induction of specific anti-STX immune responses in BALB/C mice by polyclonal murine and rabbit anti-Id

To assess the ability of the absorbed and purified murine and rabbit Ab2 in eliciting anti-STX antibody responses, groups of five BALB/C

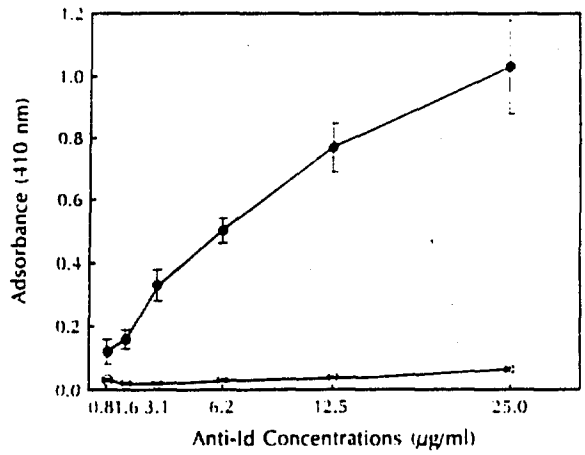

Fig. 1. ELISA reactivity of purified murine Ab2 anti-(burro-anti-STX) using microtiter wells coated with burro anti-STX (closed symbols), or with normal horse Ig (open symbols). Mean of triplicate O.D. ± SD are shown.

mice each were immunized with either unconjugated murine or rabbit Ab2, or with murine Ab2 conjugated to KLH. BALB/C mouse Ab2 unconjugated and alum hydroxide precipitated failed to elicit in BALB/C mice a significant anti-STX antibody response, even after six immunizations (data not shown). On the other hand, both the unconjugated rabbit Ab2 and BALB/C mouse Ab2 conjugated to KLH, and

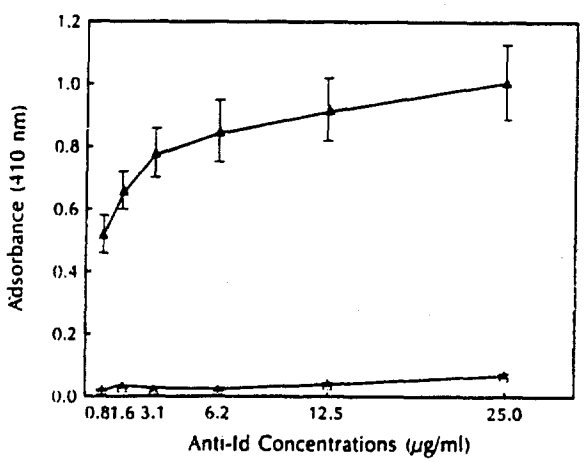

Fig. 2. ELISA reactivity of purified rabbit Ab2 anti-(burro-anti-STX) using microtiter wells coated with burro anti-STX (closed symbols) or with normal horse Ig (open symbols). Mean of triplicate O.D. ± SD are shown.

alum precipitated induced an anti-STX antibody response in BALB/C mice. All five BALB/C mice immunized with BALB/C mouse Ab2-KLH exhibited detectable anti-STX antibody reactivity in ELISA following the second immunization. The anti-STX antibody titers did not increase dramatically with subsequent immunizations; however, 2/5 and 1/5 immunized mice developed antibody tiers of 1/600 following the 7th and 8th injections, respectively (Table I). Likewise, the rabbit Ab2, unconjugated and alum precipitated, also elicited in BALB/C mice an anti-STX antibody response following the second immunization (Table II). It appeared that five to six immunizations of the rabbit Ab2 were needed to induce anti-STX titers similar to those achieved with seven to eight injections of the murine Ab2. The anti-STX immune responses induced by murine Ab2-KLH were primarily of the IgM isotype (Table I). In contrast, that elicited by immunization with the rabbit Ab2 exhibited some anti-STX IgG reactivity, although the IgM anti-STX responses were more pronounced (Table II). The anti-STX Ab3 responses in both groups appeared to be specific for the toxin antigen. The binding of pooled sera from mice immunized with murine Ab2 to STX–BSA-coated wells was efficiently inhibited by free STX (Table III). Moreover, the sera did not exhibit any significant reactivity against an unrelated toxin, namely tetrodotoxin-BSA (tdt-BSA). Similar results were obtained with sera from mice immunized with rabbit Ab2 (data not shown).

Recognition of cross-species Id by murine and rabbit anti-Id antibodies

One of the important requirements for antigenic mimicry, or internal image, of an anti-Id is its ability to recognize and bind to Ab1 raised in heterologous species against the nominal antigen (in this case STX). Thus, protein A-purified murine and rabbit Ab2 specific for burro anti-STX IgG were tested for reactivity against two murine anti-STX mAbs (S3E.2, an $IgG_{1\kappa}$, and S1A5, an IgM_{κ}) generated previously (Huot et al., 1989a). Both the murine and rabbit Ab2

TABLE I

ELISA reactivity of BALB C mice immunized with KLH-conjugated BALB C anti-(burro anti-STX)

Mouse number	Immunization no.								
	2	3	4	5	6	7	8		
							Ig	γ	μ
1,0	400[a]	400	400	800	800	800	800	10	1600
0,0	200	400	400	400	800	1600	1600	10	3200
0,1	200	400	200	800	400	800	800	80	1600
2,0	200	200	400	800	800	800	1600	40	3200
0,2	200	200	200	400	400	1600	1600	10	1600

[a] Reciprocal dilutions of sera considered positive (O.D. ≥ 0.1 and O.D. > 3 × O.D. of preimmune sera). Binding ELISA using STX-BSA-coated wells were done as previoulsy described by us (Huot et al., 1989a). A pooled serum (1:40) of mice immunized with rabbit Ab2 to an anti-mycotoxin T-2 Ab1 gave an O.D. ± SD of 0.03 ± 0.02 in this assay.

preparations showed significant binding to the two murine anti-STX mAbs, whereas no binding was observed in wells coated with normal mouse Ig or with an anti-TDT mAb (Huot et al., 1989b) (Table IV).

Anti-Id-induced anti-STX antibodies negated the protective effect of STX in cells treated with veratridine and ouabain

Treatment of murine neuroblastoma cell line CCL131 with veratridine and ouabain results in increased ion permeability, cell swelling, and eventual cell death (Gallacher and Birkbeck, 1992). In a representative experiment shown in Table V, addition of 0.25 mM veratridine and 0.5 mM ouabain resulted in approximately 77.0% neuroblastoma cell death. In the presence of STX at a concentration of 30 nM, this veratridine/ouabain-induced cytotoxicity was reduced to approximately 46.8%. When STX was preincubated with serial dilutions of pooled murine anti-STX Ab3 sera, a dose-dependent decrease in the ability of STX to protect against the cytotoxicity of veratridine and ouabain was observed. Thus, 1:10 dilution of the murine anti-STX Ab3 sera induced by mouse and rabbit Ab2 significantly inhibited the protection provided by STX, and reversed veratridine/ouabain toxicity

TABLE II

ELISA reactivity of BALB C mice immunized with rabbit anti-(burro anti-STX)

Mouse number	Immunization no.						
	2	3	4	5	6		
					Ig	γ	μ
1,0	400[a]	800	800	800	1600	160	3200
0,0	200	400	400	400	800	40	1600
0,1	800	400	800	1600	1600	1280	1600
2,0	400	800	1600	1600	3200	320	3200
0,2	200	200	800	800	800	160	800

[a] See legend of Table I.

TABLE III

Specificity of the murine anti-STX Ab3 responses induced by murine Ab2

STX-inhibitions ELISA[a]		TDT-binding ELISA[b]	
STX concentration (μg/ml)	% Inhibition	Serum dilution	O.D.
0	0	10	0.08 ± 0.03
3.1	23.6	20	0.07 ± 0.02
6.2	51.4	40	0.04 ± 0.01
12.5	86.3	80	0.03 ± 0.02
25.0	95.8	160	0.03 ± 0.01
50.0	98.2	320	0.02 ± 0.01
100.0	96.5	640	0.02 ± 0.02

[a] ELISA to assess the inhibition of binding of pooled mouse sera to STX-BSA-coated wells by different concentrations of STX was done as described by us (Huot et al., 1989a). TDT at 100 μg/ml exhibited 2.4% inhibition of the binding in this assay.

[b] TDT-binding ELISA was done with TDT-BSA-coated wells as described by us (Huot et al., 1989b). A mouse anti-TDT serum at 1:500 dilution served as the positive binding control (O.D. = 0.59 ± 0.04).

TABLE IV

ELISA reactivity of murine and rabbit Ab2 against murine anti-STX mAbs

Ab2 concentration (μg ml)	Wells coated with anti-STX mAbs	
	S3E2 IgG_1	S1A5 IgM
Murine Ab2		
5.0	0.63 + 0.08[a]	0.72 + 0.09
2.5	0.58 + 0.06	0.62 ± 0.08
1.2	0.48 + 0.04	0.53 + 0.06
0.6	0.29 + 0.04	0.32 ± 0.06
0.3	0.18 ± 0.02	0.21 ± 0.05
0.15	0.08 + 0.01	0.20 + 0.06
Rabbit Ab2		
5.0	0.85 + 0.11	1.11 + 0.13
2.5	0.79 + 0.08	0.91 + 0.10
1.2	0.67 + 0.05	0.85 + 0.09
0.6	0.48 + 0.05	0.61 ± 0.05
0.3	0.28 + 0.05	0.40 + 0.06
0.15	0.13 + 0.04	0.21 + 0.07

[a] The absorbances with anti-TDT IgG_1 mAb-coated wells were 0.09 ± 0.03 and 0.08 + 0.04 for rabbit and murine Ab2, respectively.

on CCL131 to 73.7% and 77.3%, respectively. There was no significant decrease in the STX-mediated protection by preimmune murine sera, or with a murine anti-ricin mAb (Chanh et al., 1992).

Discussion

In this manuscript, we have demonstrated the efficacy of BALB/C murine and rabbit polyclonal anti-Id antibodies specific for burro-anti-STX in eliciting a significant and specific anti-STX Ab3 response in naive BALB C mice. Since normal burro Ig was not available for adsorption studies to remove anti-burro isotypes and allotypes from the murine and rabbit Ab2 sera, the normal horse Ig-adsorbed sera may contain Ab2 with these specificities in addition to those with anti-Id reactivity. Nonetheless, administration of murine Ab2-KLH or rabbit Ab2 into naive BALB/C mice elicited detectable anti-STX Ab3 following the second immunization. Although the Ab3 response titers induced by Ab2 did not increase dramatically following subsequent immunizations with Ab2, the titers achieved with six and eight immunizations of murine Ab2-KLH and rabbit Ab2, respectively, reached dilutions of 1:800 to 1:3200. The majority of the anti-STX Ab3 responses in both groups of mice were of the IgM isotype. The anti-STX IgG titers induced by murine Ab2-KLH ranged only from 1:10 to 1:80 dilutions, whereas those induced by rabbit Ab2 were somewhat more significant, ranging from serum dilutions of 1:40 to 1:1280. The reason for this low IgG anti-STX response may be accounted for by the use of alum hydroxide instead of Freund's or other adjuvants as the immunostimulator. The observation that BALB/C murine Ab2, not conjugated to KLH protein carrier, failed to induce a significant anti-STX immune response in BALB/C mice is in concordance with previously published results suggesting the requirement for Ab2 conjugation to heterologous

TABLE V

Anti-STX Ab3 negate the protective effect of STX in veratridine ouabain treated cells

Neuroblastoma cell culture conditions			Murine anti-STX Ab3 induced by							
			Rabbit Ab2				Murine Ab2-KLH			
			PI[a]	Immune			PI	Immune		
V[b]	O[c]	STX[d]		10[e]	10	50	100	10	10	50	100
-	-	-	1.92 ± 0.2	1.90 ± 0.1[f]	1.85 ± 0.08	ND[g]	ND	1.95 ± 0.02	1.90 ± 0.1[f]	ND	ND
+	+	-	0.44 ± 0.02 (77.0)[h]	0.45 ± 0.01 (76.3)	0.46 ± 0.05 (75.1)	ND	ND	0.45 ± 0.01 (76.9)	0.47 ± 0.01 (75.3)	ND	ND
+	+	+	1.02 ± 0.10[i] (46.8)	1.1 ± 0.2[i] (42.1)	0.42 ± 0.05[i] (77.3)	0.77 ± 0.08[i] (58.4)	0.90 ± 0.06[i] (51.3)	1.2 ± 0.1[i] (38.5)	0.50 ± 0.1[i] (73.7)	0.67 ± 0.1[i] (64.7)	1.05 ± 0.2[i] (44.7)

The percent cytotoxicity, calculated as described in Materials and Methods, in the presence of ouabain veratridine STX with an irrelevant anti-ricin IgG_1 mAb was approximately 44.1%.

[a] Preimmune sera.

[b] 0.25 mM veratridine was used in the assay.

[c] 0.5 mM ouabain was used in the assay.

[d] 30 nM STX was used. This concentration usually resulted in 50% protection against cytotoxicity induced by the concentrations of veratridine and ouabain indicated above.

[e] Reciprocal dilution of sera.

[f] Mean of triplicate O.D. ± SD.

[g] Not done.

[h] Percent cytotoxicity.

[i] Significant differences at 95% confidences at 95% confidence level (p values ranging from 0.0261 to 0.002).

[j] Not significant (p values ranging from 0.382 to 0.632).

protein carriers in the induction of significant syngeneic immune responses to nominal antigens (Ward et al., 1987; Chanh et al., 1990).

The specificity of the anti-STX Ab3 responses in both groups of immunized mice were demonstrated in a number of ways. The Ab3 responses reacted with STX-BSA but not with TDT-BSA coated wells, and were specifically inhibited by free STX from binding to STX-BSA coated microtiter wells. Moreover, the anti-STX Ab3 sera were capable of negating the protective effect of STX on neuroblastoma cells treated with veratridine and ouabain, whereas an anti-ricin mAb had no effect. This property of the anti-STX Ab3 sera is presumably due to specific binding to STX, preventing it from closing the sodium channels opened by the cumulative actions off veratridine and ouabain, which result in an influx of ions inside the cells causing cellular swelling and subsequent cell death (Gallacher and Birkbeck, 1992).

We have previously demonstrated the potential use of an anti-Id mAb in eliciting a systemic anti-mycotoxin T-2 antibody response which protected against the in vitro and in vivo toxicity of T-2 (Chanh et al., 1990; 1991). The results presented herein represent, to our knowledge, the first demonstration of the use of anti-Id-based vaccine in the induction of a systemic immune response to STX, a neurotoxin. The observation that the murine and rabbit Ab2, generated by immunization with burro anti-STX IgG, recognized two previously generated heterologous anti-STX murine monoclonal antibodies suggests that some of the Ab2 may possess antigenic mimicry. Whether this anti-Id-induced anti-STX would provide protection against the in vivo toxicity of STX remains to be determined. Taken

together, these results reinforce the potential importance of anti-Id-based vaccines for the development of effective prophylaxis against non-proteinaceous, low molecular weight toxins of chemical and biological sources, whose chemical nature renders conventional approaches to vaccine development difficult at best, and whose extreme in vivo toxicity prevents their use as immunogens.

Acknowledgements

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Appendix D

ANTI-IDIOTYPE VACCINATION ELICITS PROTECTIVE IMMUNITY AGAINST THE IN VIVO TOXICITY OF SAXITOXIN

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ABSTRACT

Saxitoxin (STX) is a lethal neurotoxin which exerts its toxicity by blocking sodium ion transport across the cellular membrane. A protein G-purified burro anti-STX IgG antibody preparation was previously generated and shown to reverse the STX-mediated cardiorespiratory failure in guinea pigs. The purified burro anti-STX IgG antibodies were precipitated with aluminum hydroxide and used to immunize BALB/C mice for the production of polyclonal anti-idiotypic antibodies. The generated murine anti-idiotypic sera were rendered specific to burro anti-STX IgG antibodies by repeated absorption, and were further purified by protein A chromatography. Purified murine anti-idiotypic but not control anti-idiotypic antibodies inhibited the binding of the burro anti-STX antibodies to STX, suggesting recognition of antigenic determinant(s) closely associated with the combining site(s) of the burro antibodies. BALB/C mice immunized with the absorbed and protein A-purified syngeneic anti-idiotypic antibodies conjugated to keyhole limpet hemocyanin developed a specific humoral anti-STX antibody response which protected them against a lethal *in vivo* challenge with saxitoxin. Fifty percent of the anti-idiotype vaccinated mice survived an *in vivo* STX challenge of 8.0 μg/kg body weight, whereas all the control anti-idiotype immunized mice died. These findings further strengthen the concept of anti-idiotype-based strategy for the development of safe and effective vaccines against low molecular weight, non-proteinaceous and highly toxic compounds of biological and chemical origins.

INTRODUCTION

Saxitoxin (STX) represents one of the most lethal neurotoxin known to man with LD_{50} values (dose that kills 50% of the toxin-treated animals) in the μg/kg range.[1] Saxitoxin is a non-proteinaceous, heterocyclic guanidinium compound with an approximate molecular weight (m.w.) of 300 Da. It is a biological toxin produced by the dinoflagellates of the genus *Gonyaulax* found during ocean blooms such as the red tide.[2] The dinoflagellates are ingested in copious amounts by shellfish including clams and mussels which concentrate the toxin in their systems, without being adversely affected by the toxin.[3] However, consumption of STX-contaminated shellfish by humans may result in paralysis and even death which occurs through a rapidly progressive weakening of voluntary muscles, including respiratory muscles, resulting from the interruption of neuromuscular transmission in motor neurons[4] and in muscle fiber membranes.[5] Thus, STX contamination of shellfish during ocean blooms not only creates a safety hazard for humans, but also results in considerable economic loss to the fishing industry. The mechanism of toxicity of this paralytic toxin results from its ability to block sodium ion transport across the cell membrane.[6] Saxitoxin has been shown to bind to its specific cellular receptor with a dissociation constant of ~ 5×10^{-9} M.[7] Receptor binding studies suggest that STX cellular receptors appear to reside on the external surface of the cell membranes instead of inside the sodium channels themselves.[8] The major STX cellular receptor has been identified as a protein of ~ 260 kDa, however STX may also associate with as yet undefined smaller polypeptides.[9] Because of its chemical, structural and biological characteristics, including its low m. w. and extreme *in vivo* toxicity, it has been difficult to induce immunity against STX intoxication. Low m. w. toxins such as T-2 mycotoxin[10] and STX (T. Chanh, unpublished results) have been conjugated to different protein carriers in order to render them immunogenic, however leakage of the active form of the toxins from the toxin/carrier complexes has been demonstrated. To circumvent this difficulty, we have investigated the feasibility of eliciting active and protective immunity against STX intoxication *in vivo* by anti-idiotype (anti-Id) or Ab2 vaccination.

Immunoglobulin (Ig) molecules are characterized by three major antigenic determinants. Isotypic determinants define the class and subclass differences within a given species, whereas allotypic determinants represent intraspecies genetic polymorphism. Idiotypic (Id) determinants are defined as amino acid sequences associated with the variable (V) region of the Ig molecule. One of the postulates of the network theory formulated by Jerne is that some anti-Id antibodies (or Ab2), termed internal image anti-Id or Ab2β whose binding to the Id determinants can be specifically inhibited by the antigens, have the ability to serologically and structurally mimic the nominal antigens.[11] This "antigenic mimicry" has led to the hypothesis that anti-Id antibodies may have important applications in vaccine development.[12] Indeed, ample experimental evidence exist in support of the ability of anti-Id antibodies to mimic antigens and to induce specific immune responsiveness in a variety of antigenic systems.[13-28] Our laboratories have been interested in the anti-Id antibody-based approach for the development of effective vaccines against a variety of low m.w. toxins of biological and chemical origins. We have previously reported the induction of active, specific and protective immunity against the *in vivo* toxicity of T-2 mycotoxin (a protein synthesis inhibitor) by vaccination of syngeneic mice with a BALB/C anti-Id monoclonal antibody (mAb) specific for a protective anti-T-2 mAb.[29-31] Polyclonal Ab2 antibodies to goat anti-ricin antibodies have also been generated and demonstrated to induce in syngeneic and xenogeneic species specific anti-ricin antibody responses which effectively protected the EL-4 murine myeloma cell line against the *in vitro* cytotoxicity of ricin.[32] Herein, we report that BALB/C mice vaccinated with syngeneic Ab2 anti-Id antibodies to the burro anti-STX antibodies developed a specific and active antibody response which protected them from a lethal *in vivo* STX challenge. The results presented herein and previously published findings discussed above suggest that the anti-Id vaccine approach may represent a safe and effective mean for the elicitation of active and protective immunity against low m. w. and highly toxic compounds of biological origin.

MATERIALS AND METHODS

Burro anti-STX IgG antibodies

The IgG fraction of the burro anti-STX antibodies was obtained by protein G chromatography and was previously demonstrated to reverse the STX-induced cardiorespiratory failure in guinea pigs.[33]

Generation of BALB/C anti-idiotype Ab2 sera.

The protein G-purified burro anti-STX IgG antibodies were precipitated with aluminum hydroxide as described earlier.[34] Female BALB/C mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were immunized i.p. with 50 μg/injection of the aluminum hydroxide precipitated burro anti-STX IgG every other week. The mice were bled two weeks subsequent to the fourth immunization, and their sera were pooled and stored at -80°C for analysis. To remove anti-allotype and anti-isotype reactivity, the murine sera were absorbed repeatedly over agarose conjugated to normal horse Ig (NHIg, Sigma Chemical Co., St. Louis, MO). Normal horse Ig was used for this purpose because it is more readily available than normal burro Ig and because there is good cross-reactivity between these two species' Ig as a result of their close phylogenetic relationship. The murine Ab2 antibodies were further purified by protein A chromatography, as described elsewhere[35], for further analysis.

ELISA for the detection of BALB/C mouse anti-Id antibodies

Enzyme-linked immunosorbent assays (ELISAs) were used to detect BALB/C mouse Ab2 antibody reactivity to burro anti-STX antibodies. Microtiter wells were coated with 0.05 ml of a predetermined optimum concentration of 2.0 μg/ml of NHIg or burro anti-STX IgG antibodies overnight at 4°C. The wells were washed with phosphate buffered saline (PBS, pH 7.2) supplemented with 1% Tween-20 (wash buffer), and blocked with PBS containing 5% normal goat serum (PBS/NGS) for 1 hr at 37°C. After washing, 0.05 ml containing different concentra-

tions of the absorbed and protein A-purified murine Ab2 antibodies were added to the wells which were incubated for 1 hr at 37°C. Pooled preimmune murine Ig and BALB/C mouse anti-Id Ab2 antibodies specific for goat anti-ricin IgG antibodies, purified as above, were used as negative controls.[32] After incubation and washing, 0.05 ml of a 1:2000 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase (HRP, Fisher Scientific, Orangeburg, NY) were added to the wells. Reactivity was developed by the addition of the substrate 2,2-azino-bis-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) dissolved in 0.3% 0.1M citric acid buffer, pH 4.0 (Sigma Chemical Co.). The reaction was stopped by the addition of 5% SDS in PBS, and absorbance was determined at 410 nm with a Dynatech microplate reader. All determinations were done in triplicate.

Inhibition of burro anti-STX binding to STX by murine anti-Id antibodies

The protein A-purified murine Ab2 antibodies were tested for their ability to inhibit the binding of the burro anti-STX IgG to STX as follows. Equal volume (0.05 ml) of 1.0 μg/ml of burro anti-STX conjugated to biotin[34] was admixed with different concentrations of the purified murine Ab2 antibodies for 30 min at 37°C. The mixtures were added to microtiter wells coated with 10 μg/ml of STX-BSA conjugate prepared as we previously described.[36] After 1 hr of incubation at 37°C, binding or inhibition thereof was detected by the addition of avidin-HRP and the substrate, ABTS.[36] Pooled and protein A-purified preimmune mouse IgG and mouse anti-Id IgG to goat anti-ricin were used as negative controls.

Vaccination of BALB/C mice with BALB/C anti-Id

The protein A-purified murine Ab2 IgG antibodies to burro anti-STX antibodies(and the control murine Ab2 anti-Id to goat anti-ricin antibodies) were conjugated to keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC, Sigma Chemical Co.), as previously described by us.[31] Groups of BALB/C mice were vaccinated i.p. every three weeks with 50μg/injection of the syngeneic murine Ab2 anti-Id antibodies. The Ab2 anti-Id antibodies were emulsified in Freund's complete adjuvant (FCA) for the first injection, Freund's incomplete adjuvant (FIA) for the second booster injection, and in PBS for

subsequent immunizations. The mice were bled two weeks after the fifth injection, and their sera were tested for ELISA reactivity as described below. Other groups of BALB/C mice were similarly immunized and challenged with different doses of STX, as described below.

Detection of anti-STX antibody responses induced by anti-Id immunization

Serial dilutions of the murine anti-anti-Id-antibodies (Ab3) were tested for reactivity using microtiter wells coated with STX-BSA[36] or tetrodotoxin- (TDT) BSA[37], as we previously described. Inhibition of binding was also assessed using a 1:500 dilution of pooled immune mouse sera obtained two weeks after the fifth injection, and either STX (USAMRIID, Fort Detrick, Frederick, MD) or TDT (Sigma Chemical Co.) as the inhibitor to further determine the specificity of the anti-Id-induced anti-STX antibody responses.[36,37]

In vivo STX challenge

After the fifth Ab2 anti-Id immunization, groups of mice were challenged s.c. with various doses of STX in 0.1 ml of sterile PBS. The number of mice that died from the STX challenge, and the time between challenge and death (elapsed time) were recorded.

RESULTS

Specificity of the absorbed and purified BALB/C mouse anti-Id antibodies

BALB/C mice vaccinated with the protein G-purified burro anti-STX IgG precipitated in aluminum hydroxide were bled two weeks subsequent to the fourth immunization. Their sera were pooled and absorbed by repeated passages over NHIg-agarose column, and purified by protein A chromatography. Following the third absorption run over NHIg-agarose column, the BALB/C mouse Ab2 anti-Id antibodies still exhibited residual ELISA reactivity against NHIg-coated microtiter wells (data not shown). However, after the fourth absorption run, the murine Ab2

preparation showed no remaining reactivity with NHIg even at a concentration of 50 μg/ml, but still reacted strongly with the burro anti-STX IgG (Table 1), with an approximate 50% binding end-point at a concentration of ~ 3.1 μg/ml.

Murine anti-Id antibodies inhibited the binding of burro anti-STX to STX

To determine whether the Id determinant(s) recognized by the absorbed and purified murine Ab2 anti-Id antibodies was associated with the STX-binding site(s) of the burro anti-STX IgG, inhibition ELISAs designed to assess the ability of the purified murine Ab2 antibodies to inhibit the binding of the burro anti-STX antibodies to STX-BSA-coated wells were performed. Burro anti-STX IgG antibodies preincubated with the control purified murine Ab2 antibodies to goat anti-ricin antibodies were still capable of binding to STX-BSA coated wells (Table 2). In addition, neither of the preimmune murine Ig inhibited the binding of the burro anti-STX antibodies to STX-BSA. Conversely, the murine Ab2 antibodies raised to burro anti-STX antibodies significantly inhibited the latter from binding to STX-BSA in a dose-dependent fashion (Table 2). Concentrations of murine Ab2 antibodies equal to or higher than 1.2 μg/ml inhibited the binding of the burro anti-STX antibodies to STX-BSA by greater than 93%. An approximated 50% inhibition of binding end-point was achieved with a concentration of the BALB/C mouse Ab2 antibodies between 0.6 μg/ml and 0.3 μg/ml.

Murine anti-Id antibodies elicited specific and protective anti-STX immune responses in vivo

The specificity of the anti-Id-induced anti-STX antibody responses in a group of five BALB/C mice immunized with syngeneic anti-Id antibodies was assessed by ELISAs. The binding of the Ab2-induced antibody responses to STX-BSA-coated microtiter wells was inhibited by STX, but not by the structurally related Na^+ channel blocker, TDT (Table 3). STX at a concentration of 6.2 μg/ml inhibited this binding by approximately 48.9%, whereas 100 μg/ml of TDT exhibited only 2.2% inhibition of binding. Concentrations of STX greater than 25 μg/ml exhibited greater than 95% of the binding. The Ab2-induced murine antibody responses were also shown to bind to STX-BSA- but not to TDT-BSA-coated microtiter wells (data not shown). These results led us to

similarly immunize a larger group of BALB/C mice for *in vivo* protection experiments. After the fifth vaccination with absorbed and protein A-purified BALB/C Ab2 anti-Id to burro anti-STX or to goat anti-ricin, the mice were challenged with different doses of STX (Table 4). No apparent protection was observed with the STX challenge dose of 16 μg/kg body weight as all toxin-treated mice succumbed to STX toxicity. On the other hand, some protection was observed with the group of mice challenged with 12 μg/kg body weight of STX. One out the 10 mice immunized with murine anti-Id antibodies to burro anti-STX antibodies survived this STX challenge dose, whereas both mice vaccinated with the control anti-Id antibodies died. Furthermore, some delay in the onset of death was also observed in some mice in the group vaccinated with Ab2 anti-Id antibodies to burro anti-STX, as compared to the group immunized with the control Ab2 anti-Id (Table 4). The protection became more apparent with the STX dose of 8.0 μg/kg body weight as 5 out of the 10 mice (50%) vaccinated with Ab2 antibodies to burro anti-STX survived, whereas none of the control mice did.

DISCUSSION

The structural and biological properties of the Na^+ channel blocker STX render it unsuitable as a conventional immunogen for the elicitation of protective immunity against its lethal toxicity. Antibodies specific for STX have been previously generated employing STX conjugated to KLH.[36] However, this may not represent a safe approach since conjugation of T-2 mycotoxin[10] and STX (T. Chanh, unpublished observation) to large protein carriers for the purpose of enhancing immunogenicity, has been shown to result in unstable toxin:carrier complexes with potential release of the active toxins from the complexes. Therefore, we have undertaken studies to assess the feasibility of employing the Ab2 anti-Id-based approach in eliciting immunity against STX *in vivo* toxicity. Polyclonal BALB/C mouse Ab2 antibodies were produced by immunization with burro anti-STX IgG antibodies purified by protein G chromatography which had been shown to reverse the *in vitro* effects of STX.[33] The resulting murine Ab2 sera, obtained two weeks following

the fourth immunization, were pooled and rendered specific for the burro anti-STX antibodies by repeated absorption, and Ab2 IgG antibodies were purified by protein A chromatography. The murine Ab2 antibodies appeared to recognize Id determinant(s) closely associated with the STX-binding site(s) of burro anti-STX antibodies since they efficiently inhibited the binding of the burro antibodies to STX-BSA-coated microtiter wells (Table 2).

The purified BALB/C mouse Ab2 antibodies were conjugated to KLH and used to vaccinate syngeneic mice for the induction of specific anti-STX antibody responses. We and others had demonstrated previously that in syngeneic systems, conjugation of Ab2 antibodies to heterologous protein carriers was required for the induction of significant antibody responses to the nominal antigens.[18,31] Vaccination of naive syngeneic mice with the Ab2 anti-Id antibodies was shown to elicit a specific anti-STX antibody response (Table 3). The Ab2-induced anti-STX antibody response was specific as shown by the ability of free STX, but not TDT, to inhibit its binding to STX. This anti-STX immune response was also demonstrated to protect mice from *in vivo* challenge with STX (Table 4). Although all the vaccinated mice died from the highest challenge dose of STX used (16.0 μg/kg body weight), various degree of protection was obtained with the lower STX challenge doses as assessed by both the survival ratio (number of surviving mice/total number of mice) and the elapsed time between STX administration and death. One of ten mice vaccinated with murine Ab2 to burro anti-STX and challenged with 12 μg/kg STX survived, whereas all control Ab2 anti-Id immune mice died. In addition, a considerable delay in the time of death following STX administration was observed in some of the mice immunized with Ab2 antibodies to burro anti-STX and challenged with 12 μg/ml STX, as compared to that of the control group. At the lowest STX challenge dose used (8.0 μg/kg body weight), 5 out of 10 (50%) of the Ab2-immune mice survived, whereas all the control mice succumbed to the STX challenge (Table 4). It should be pointed out that in previous *in vivo* protection experiments, control mice always succumbed to challenge with the dose range of STX used in these studies.

Theoretically, it should be possible to design effective Ab2 anti-Id vaccines against all toxic chemicals with the provision that sufficiently high-affinity binding antitoxin antibodies (Ab1) can be generated that can compete with the target sites for binding to and neutralization of circulating

toxins before they reach lethal levels in blood. This requirement represents one of the drawbacks of the anti-Id approach in toxicology. Antigen/antibody interactions are primarily mediated by noncovalent ionic forces with relative binding constants ranging between 10^{-6} M and 10^{-9} M. This property imposes a limit on antibody-based protection against toxic substances possessing higher binding affinity constants for their target sites than those of antibody/toxin interactions.

Nevertheless, the experimental results presented herein strengthen our previously reported studies of Ab2-induced protective immunity in toxicology[38,39], and support the concept that the Ab2 antibody-based approach may represent the only safe alternative to vaccine development strategy against low molecular weight, nonproteinaceous and highly toxic compounds.

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Vertebrate Animal Use: The research reported herein was conducted in accordance to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, HHs Pub. No. (NIH) 85-23, revised 1985. All described experimental protocols involving animals have been approved by the Institutional Animal Care and Use Committee (IACUC).

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TABLE 1

Binding specificity of the BALB/C anti-Id antibodies to burro anti-STX IgG

BALB/C anti-Id (μg/ml)	ELISA reactivity with	
	Burro anti-STX IgG	Normal horse IgG
50.0	0.98 ± 0.08[a]	0.07 ± 0.04
25.0	1.02 ± 0.10	0.05 ± 0.03
12.5	0.77 ± 0.06	0.08 ± 0.04
6.2	0.55 ± 0.06	0.06 ± 0.03
3.1	0.49 ± 0.03	0.03 ± 0.02
1.6	0.29 ± 0.04	ND[b]
0.8	0.12 ± 0.03	ND
0.4	0.08 ± 0.04	ND

The mice were bled after the fourth immunization with burro anti-STX IgG antibodies. Their pooled sera were absorbed and purified as described in Materials and Methods.

[a]Mean OD at 410 nm ± SD.

[b]Not done.

TABLE 2

BALB/C anti-Id antibodies inhibit burro anti-STX binding to STX-BSA-wells

Mouse antibodies (μg/ml)	Binding of burro anti-STX in the presence of anti-Id to			
	Burro anti-STX		Goat anti-ricin	
	Preimmune	Immune[a]	Preimmune	Immune
5.0	0.81 ± 0.06[b]	0.07 ± 0.03 (94.9)[c]	0.82 ± 0.09	0.80 ± 0.08
2.5	0.80 ± 0.09	0.06 ± 0.04 (96.1)	0.79 ± 0.10	0.81 ± 0.07
1.2	0.78 ± 0.08	0.08 ± 0.04 (93.3)	0.80 ± 0.07	0.77 ± 0.09
0.6	0.80 ± 0.07	0.29 ± 0.06 (66.2)	ND[d]	0.82 ± 0.08
0.3	0.78 ± 0.09	0.48 ± 0.04 (40.0)	ND	0.75 ± 0.07
0.15	0.81 ± 0.08	0.65 ± 0.06 (20.5)	ND	0.78 ± 0.07
0.07	0.78 ± 0.07	0.75 ± 0.08 (2.6)	ND	0.79 ± 0.06

[a] Purified antibodies were prepared from absorbed mouse sera obtained two weeks after the fourth injection of burro anti-STX antibodies.

[b] Mean OD at 410 nm ± SD.

[c] The values in parenthesis represent the percent inhibition of binding calculated as $[(PI_{OD} - Bckgrd_{OD}) - (Pol_{OD} - Bckgrd_{OD})] \div (PI_{OD} - Bckgrd_{OD}) \times 100]$, where PI_{OD}, Pol_{OD}, and $Bckgrd_{OD}$ represent the OD values of preimmune, postimmune and background, respectively.

[d] Not done

TABLE 3

Specificity of the anti-STX immune response induced by anti-idiotypes

Inhibitor Concentration (μg/ml)	Reactivity of anti-Id-induced anti-STX response with STX-BSA-coated wells in the presence of inhibitor	
	Saxitoxin	Tetrodotoxin
0	0.45 ± 0.06 (0)[a]	0.47 ± 0.05 (0)
3.1	0.35 ± 0.04 (22.2)	0.46 ± 0.04 (0)
6.2	0.23 ± 0.03 (48.9)	0.45 ± 0.05 (0)
12.5	0.09 ± 0.03 (80.0)	0.48 ± 0.07 (0)
25.2	0.015 ± 0.03 (96.7)	0.43 ± 0.06 (4.4)
50	0.021 ± 0.03 (95.3)	0.45 ± 0.08 (0)
100	0.017 ± 0.04 (96.2)	0.44 ± 0.06 (2.2)

The inhibition ELISAs were done as previously described.[36,37] Mouse sera obtained after the fifth injection of syngeneic anti-Id were pooled and used at a dilution of 1:500.

[a]Mean OD at 410 nm ± SD. The values in parenthesis represent the mean percent inhibition of binding of triplicates determined as [(mean OD without inhibitor - OD_{bckgrd}) - (mean OD with inhibitor - OD_{bckgrd}) / (mean OD without inhibitor - OD_{bckgrd})] x 100.

TABLE 4

Anti-idiotype-induced protection against STX challenge *in vivo*

STX (μg/kg)	Survival ratio[a]		Elapsed time (min)[b]	
	Immunization with anti-Id to			
	Burro anti-STX	Goat anti-ricin	Burro anti-STX	Goat anti-ricin
16.0	0/5	0/2	3:10; 3:14 3:18; 3:31 3:48	3:18; 4:03
12.0	**1/10**	0/2	4:26; 4:34 5:07; 5:32 5:54; 7:08 8:16; 10:08 11:54; one mouse survived	5:12; 5:24
8.0	**5/10**	0/2	5:58; 6:18 7:25; 9:21 9:30; five mice survived	6:48; 8:40

[a] Number of surviving mice / total number of mice.

[b] Time in minutes between STX challenge and death.

Appendix E

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MONOCLONAL ANTIBODY PROPHYLAXIS AGAINST THE *IN VIVO* TOXICITY OF RICIN IN MICE

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ABSTRACT

A BALB/c murine IgG_1 monoclonal antibody, designated BG11-G2, specific for ricin was generated. BG11-G2 antibody did not bind to either purified ricin chain A or chain B, but recognized an antigenic determinant whose conformation requires the combination of the two chains in the formation of the native ricin molecule. It did not react with the protein synthesis inhibitor, T-2 mycotoxin, or with the sodium channel blockers, saxitoxin and tetrodotoxin. As little as 0.78 μg/ml of BG11-G2 IgG_1 anti-ricin monoclonal antibody completely protected against the *in vitro* toxicity of ricin as determined by [^{3}H]leucine uptake in EL-4 cell assays. Passive intraperitoneal infusion of purified BG11-G2 antibody into BALB/c mice one day prior to a lethal challenge with ricin considerably delayed the onset of toxicity and death. Better protection was obtained with BG11-G2 infused before and after ricin challenge.

INTRODUCTION

Discovered in the 1800's, the protein synthesis inhibitor, ricin, produced by the seeds of the castor plant, Ricinus communis, represents one of the most toxic chemicals known to man. The ricin molecule is a disulfide-bonded glycoprotein heterodimer consisting of a 32 kDa A chain linked to a 32 kDa B chain (1). The two ricin chains appear to have strong affinity for one another, and association of the two chains is required for maximum toxicity. The ricin B chain promotes binding of the ricin molecule to cellular membranes through its binding specificities for complex galactosides (2,3). This binding to cell surface receptors is believed to trigger the endocytic uptake of ricin molecules (4) and to facilitate the intracellular escape of the A chain from endosomes into the cytoplasm where toxicity takes place (5). Once inside the eukaryotic cell cytoplasm, ricin chain A quickly mediates an enzymatic attack on the cellular protein synthesis at the level of the 60S ribosomal subunit (6). Although the exact mechanism of protein synthesis inhibition remains unclear, studies have shown that ricin-treated ribosomes lack an adenine at position A_{4324}, although the phosphoribose backbone remains intact (7). It

was further shown that ricin removes only one adenine residue per ribosome subunit, suggesting that A_{4324} may represent the only site of action of ricin toxicity (7).

Although monoclonal antibodies (mAb) specific for ricin have been generated, their potential abilities to protect against ricin toxicity have not been investigated (8). Herein, we describe the generation and characterization of BG11-G2 murine mAb specific for an antigenic determinant associated with the intact ricin molecule. This mAb was shown to be effective in protecting against the *in vitro* cytotoxicity of ricin, and in delaying the onset of ricin toxicity and death in mice.

MATERIALS AND METHODS

Mouse immunization and monoclonal antibody generation

Three- to five-week-old female BALB/c mice were immunized subcutaneously on a bi-weekly schedule with a sublethal dose (0.1 μg/mouse) of whole ricin (Sigma Chemical Co., St. Louis, MO). The primary immunization was administered in Freund's complete adjuvant (FCA), followed by Freund's incomplete adjuvant (FIA). Subsequent immunizations were given in phosphate buffered saline (PBS, pH 7.3). Six days after the fourth booster injection, the mice were sacrificed, and a single spleen cell suspension was prepared and fused with the murine myeloma cell line Sp2/0 essentially as described previously (9).

Detection of monoclonal anti-ricin antibodies

Hybrid culture supernatant reactivity against ricin was detected using an enzyme-linked immunosorbent assay (ELISA). In this assay, microtiter wells (Corning Glass Works, Ithaca, NY) were precoated with 0.05 ml/well of a predetermined concentration of ricin (10 μg/ml) in PBS, and incubated at 4°C overnight. The microtiter wells were washed twice with washing buffer composed of 10% Tween-20 in PBS. Unreacted sites on the wells were blocked with PBS containing 5% normal goat serum (PBS/NGS) for 1 hr at 37°C. After blocking, the wells were washed and 0.05 ml of hybrid culture supernatants were added to the wells, which were incubated for 1 hr at 37°C. After incubation, the wells were washed with washing buffer and reacted with 0.05 ml of a 1:2500 dilution of goat anti-mouse immunoglobulin (Ig) conjugated to horseradish peroxidase (HRP) (Fisher Scientific, Pittsburgh, PA). After 1 hr incubation at 37°C and washing, reactivity was detected by adding the substrate 0.3% 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate in 0.1 M citric acid buffer, pH 4.0 (ABTS, Sigma Chemical Co.). The optical density (O.D.) was determined at a wavelength of 410 nm using a microplate reader (Dynatech Laboratories, Alexandria, VA). The ricin-immune mouse serum at a 1:250 dilution served as the positive control, whereas an IgG_1 mAb specific for T-2 mycotoxin served as the negative control (9). Determinations were done in duplicate.

Generation of ascites and purification of mAb

Ascitic fluid was generated by injecting pristane-primed BALB/c mice with 1 to 5 x 10^6 BG11-G2-secreting hybridoma cells. The harvested ascites was cleared by centrifugation, and purified by a combination of caprylic acid and saturated ammonium sulfate precipitation essentially as described previously (10). Antibody purity was assessed by SDS-PAGE analysis as described (11).

Specificity analysis

The reactivity of BG11-G2 mAb against various toxin antigens was determined in binding ELISA with microtiter wells coated with either whole ricin as described above, or 10 μg/ml of purified ricin chain A, or chain B (Sigma), or T-2 mycotoxin conjugated to bovine serum albumin (T-2-BSA) (9), or saxitoxin-BSA (STX-BSA) (12), or tetrodotoxin-BSA (TDT-BSA) (13) as described. Culture supernatants of the murine hybridoma cell lines TFTA-1 and TFTB-1 specific for ricin chains A and B, respectively, were used as positive controls (American Type Culture Collection, Rockville, MD) (14). An inhibition ELISA was also performed in which a predetermined concentration of BG11-G2 mAb (30 ng/ml) that gave approximately 40-60% binding to ricin-coated wells was incubated for 1 hr at 37°C with various concentrations of inhibitors (ricin, ricin chain A or chain B, T-2, TDT, or SXT). The mAb/inhibitor mixtures were added to ricin-coated wells, and the ELISA was done as described above.

Antibody isotype

Monoclonal antibody isotyping was performed using both an antigen-dependent and an antigen-independent ELISA. In the antigen-dependent ELISA, the assay was done as above with substitution of the goat anti-mouse Ig-HRP with goat IgG specific for mouse Ig isotypes conjugated to HRP (Fisher Scientific). For the antigen-independent ELISA, an isotyping kit was used according to the manufacturer's specifications (Bio-Rad, Richmond, CA).

Ricin *in vitro* toxicity

The murine myeloma EL-4 cell line was purchased from the American Type Culture Collection (Rockville, MD). The EL-4 cells in log-phase growth were plated in 96-well, flat bottom tissue culture plates at a density of 3 x 10^5/well in RPMI 1640 (Cell Culture Laboratories, Cleveland, OH) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) and allowed to incubate overnight. To determine the concentration of ricin that kills approximately 50% of the EL-4 cells (IC_{50}), various concentrations of ricin (ranging from 1.0 ng to 100 ng) were added to triplicate EL-4 wells. The wells were incubated for 3 hr at 37°C, and washed with sterile PBS, and leucine-deficient culture medium supplemented with [^{3}H]leucine (1.0 μCi/well) (New England Nuclear, Boston, MA) was added. The wells were incubated for another 3 hr, harvested, and processed for scintillation counting. The percent inhibition (%I) of [^{3}H]leucine uptake by EL-4 cells was determined as [(mean cpm without ricin - mean cpm of

background) - (mean cpm with ricin - mean cpm background)| / |(mean cpm without ricin - mean cpm of background)| x 100. In our hands, the ricin IC_{50} value was approximately 6.25 ng/ml under the conditions described above.

Protection against ricin *in vitro* toxicity

To assess the ability of the mAb to protect against the *in vitro* toxicity of ricin, various concentrations of purified BG11-G2 mAb were incubated for 2 hr with an equal volume of twice the amount of ricin that inhibits 50% of the [^{3}H]leucine uptake as determined above. The ricin/mAb mixtures were then added to triplicate wells and the cytotoxicity assay was performed essentially as described above. The HD11 IgG_1 anti-T-2 mycotoxin mAb served as the negative antibody control.

Passive protection against the *in vivo* toxicity of ricin

Groups of BALB/c mice were infused intraperitoneally with purified BG11-G2 mAb in PBS (10 mg/ml). The following day (approximately 18 hr post-infusion), the mice were challenged subcutaneously with different amounts of ricin. The number of mice that died of ricin toxicity and the time intervals between challenge and death were recorded. In another experiment, groups of BALB/c mice were infused with BG11-G2 mAb approximately 18 hr before ricin challenge followed by antibody infusions 30 min, 24 hr, and 72 hr post-challenge.

RESULTS

Characterization of BG11-G2 monoclonal anti-ricin antibody

BALB/c mice immunized with a sublethal dose (0.1 μg/ml) of ricin developed detectable serum anti-ricin antibody activity following the second immunization (data not shown). After mice had received four injections, their spleen mononuclear cells were fused with the murine myeloma cell line Sp2/0, and the resulting hybrid cell culture supernatants were screened for anti-ricin reactivity by ELISA as described in the Materials and Methods section. Twenty (3.1%) out of 650 hybrids screened had reactivity against ricin. Among these 20 hybrids, one (BG11-G2) was found in preliminary EL-4 assay to protect against the *in vitro* toxicity of ricin (data not shown), and was selected for further studies. BG11-G2 mAb reacted in ELISA with microtiter wells coated with intact ricin, but not with the unrelated toxins T-2 mycotoxin, saxitoxin (STX) or tetrodotoxin (TDT) (Table I). Moreover, no reactivity was observed with wells coated with purified ricin chains A or B.

The BG11-G2 hybrid cells were subcloned by limiting dilution until greater than 90% of the derived cultures were antibody-positive. Monoclonal BG11-G2 anti-ricin antibody is an IgG_{1k} mAb, as determined by both the antigen-dependent and antigen-independent isotyping assays

TABLE I

ELISA reactivity of BG11-G2 antibody

BG11-G2 dilution	Microtiter wells coated with					
	Ricin	A chain	B chain	T-2	SXT	TDT
Neat	0.83[a]	0	0	0	0	0
1:2	0.80	0	0	0	0	0
1:4	0.86	0	0	0	0	0
1:8	0.75	0	0	0	0	0

[a]Mean optical density (410 nm) of duplicate determinations minus background values obtained with mAbs of irrelevant specificity.

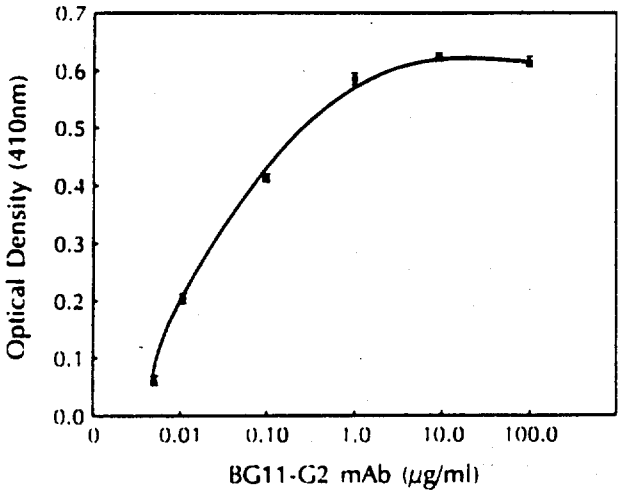

FIGURE 1

Titration of BG11-G2 mAb binding to ricin in ELISA. Each point represents the mean of duplicate determinations. The bars represent the range of duplicates.

(data not shown). Ascites was produced by i.p. injection of BG11-G2 hybrid cells into BALB/c mice, from which BG11-G2 IgG_1 mAb was purified by saturated ammonium sulfate and caprylic acid precipitation, as described. The purified BG11-G2 anti-ricin mAb was titrated for binding to whole-ricin-coated microtiter wells (Fig. 1). Maximum binding was obtained with BG11-G2 concentrations equal to or greater than 1.0 μg/ml. The 50% binding endpoint was approximated from the binding curve to be between 30 and 40 ng/ml of BG11-G2. The specificity of BG11-G2 mAb was further tested in inhibition ELISA using whole ricin, or isolated ricin chain A or chain B as inhibitors. Whole ricin was able to inhibit the binding of BG11-G2 in a dose-dependent fashion (Fig. 2). The 50% inhibition end-point was achieved with approximately 0.75 μg/ml of ricin. No

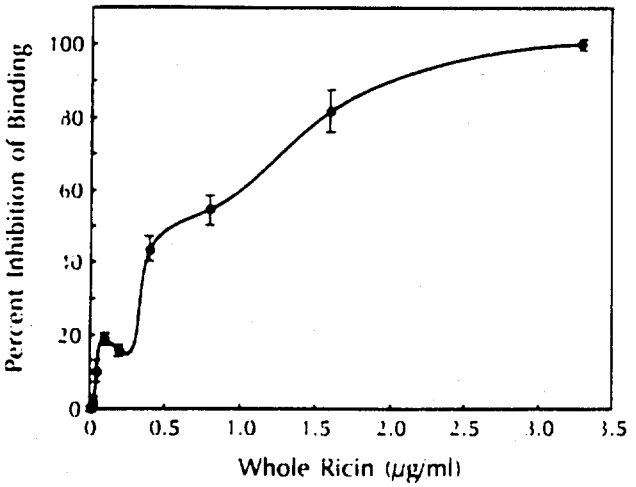

FIGURE 2

Inhibition of BG11-G2 mAb binding to ricin-coated microtiter wells by different concentrations of ricin. See legend of Figure 1.

inhibition of binding was observed with purified ricin chains A and B nor with the irrelevant T-2, TDT, and SXT toxins at a concentration of 200 μg/ml (data not shown).

BG11-G2 mAb protects against the *in vitro* toxicity of ricin

To assess the protective capacity of BG11-G2 against the *in vitro* toxicity of ricin, we determined the [^{3}H]leucine uptake by EL-4 cells in the presence of ricin (IC_{50} of 6.25 ng/ml) with or without addition of BG11-G2 mAb (Table II). In the presence of 6.25 ng/ml of ricin alone, the [^{3}H]leucine uptake by EL-4 cells was inhibited by approximately 47%. Addition of BG11-G2 anti-ricin mAb at concentrations equal to or greater than 0.78 μg/ml completely reversed this inhibition. Lower concentrations of BG11-G2 resulted in a dose-dependent protection of EL-4 cells against ricin toxicity. Addition of 10 μg/ml of HD11 anti-T-2 mycotoxin IgG_1 mAb had no protective effect.

Passive protection against ricin *in vivo* toxicity by BG11-G2

The LD_{50} (dose that kills 50% of challenged mice) in BALB/c mice administered ricin either intramuscularly or subcutaneously is approximately 0.5 μg/mouse in our hands (data not shown). To test for BG11-G2 mAb protection against the *in vivo* toxicity of ricin, groups of five BALB/c mice each were infused with 10.0 mg/ml of purified BG11-G2 intraperitoneally. The following day (approximately 18 hr post-infusion), the mice were challenged subcutaneously with either 2.5 μg or 5.0 μg/mouse of ricin (Fig. 3). Although all mice eventually succumbed to ricin

TABLE II

BG11-G2 anti-ricin antibody protects against the *in vitro* toxicity of ricin.

Antibody (μg/ml)	Without ricin	With ricin[a]	%I[b]
None	45,142 ± 3,789[c]	23,856 ± 3,004	47.15
BG-11-G2:			
(1.56)	43,671 ± 2,945	45,890 ± 4,109	0
(0.78)	45,324 ± 3,109	46,783 ± 3,814	0
(0.39)	43,783 ± 4,015	37,525 ± 3,164	14.29
(0.19)	48,120 ± 3,867	41,524 ± 4,569	13.71
(0.097)	40,786 ± 4,091	31,248 ± 3,019	23.38
(0.048)	47,094 ± 4,365	26,154 ± 2,683	44.46
HD11			
(10)	41,436 ± 3,902	20,017 ± 4,013	51.69

[a]Ricin used was 6.25 ng/ml.
[b]Mean percent inhibition of [^{3}H] leucine uptake was determined as described in Materials and Methods.
[c]Mean triplicate cpm ± SE.

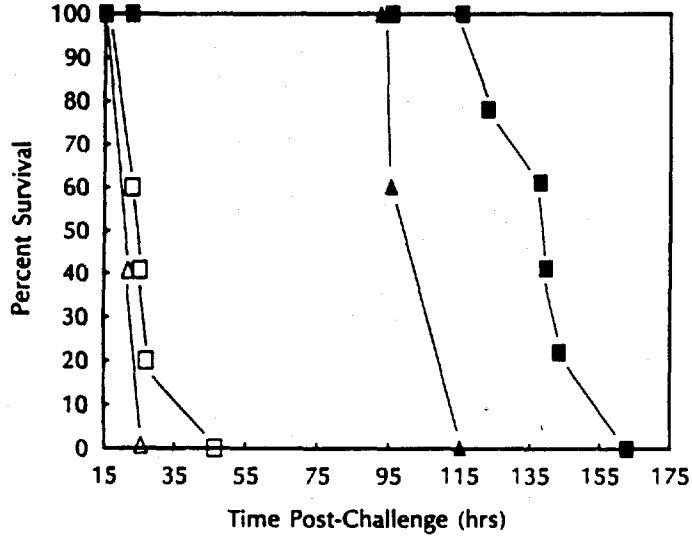

FIGURE 3

Protection against ricin *in vivo* toxicity by infusion of BG11-G2 mAb before challenge with 2.5 μg (squares) or 5.0 μg (triangles) of ricin. Open symbols represents untreated mice, whereas closed symbols represents mice infused with BG11-G2 mAb.

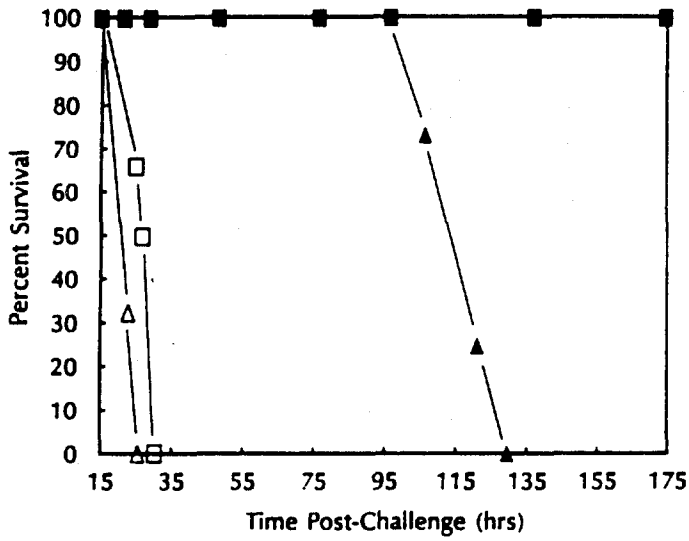

FIGURE 4

Protection against ricin *in vivo* toxicity by infusion of BG11-G2 mAb before and after challenge with ricin. See legend in Figure 3.

challenge, there was a considerable delay of the onset of death in mice treated with BG11-G2 mAb compared to those not treated. Thus, while all untreated mice challenged with 2.5 μg of ricin died about 48 hr following ricin administration, BG11-G2-treated mice survived for up to 165 hr post-ricin challenge. All untreated mice challenged with 5.0 μg ricin/mouse died approximately 25 hr after ricin challenge. On the other hand, it took 115 hr for all the mAb-treated mice to succumb to this dose of ricin. No protection was observed when control mice were infused with a specific anti-ricin mAb that did not protect against the *in vitro* toxicity of ricin in EL-4 cell assays (data not shown).

In another experiment, groups of four mice each were infused with 10 mg/ml of BG11-G2 mAb 18 hr before challenge with 4.0 μg or 2.0 μg ricin/mouse. The mice were also given post-challenge infusions of BG11-G2 mAb at the time points indicated in Materials and Methods. Again, a rapid onset of death (23-32 hr post-challenge) was observed in the groups of mice that received 2.0 or 4.0 μg ricin/mouse, but were not treated with BG11-G2 mAb (Figure 4). In contrast, 100% of the mAb-treated mice challenged with 4.0 μg of ricin survived 95 hr post-challenge; however, they all succumbed approximately 115 hr post-challenge. On the other hand, all four BG11-G2-treated mice challenged with 2.0 μg ricin/mouse survived the lethal challenge with ricin and appear to be completely normal at this writing (approximately 3 months post-ricin-challenge).

DISCUSSION

Ricin represents one of the most studied and most potent protein synthesis inhibitors known. Because of its extreme toxicity, a great deal of interest has been placed on the development of immunotoxins containing ricin. One of the most common ways to prepare immunotoxins is the covalent linkage of the purified toxic ricin A chain to a specific mAb (14,15). This strategy results in a reduction of some of the nonspecific toxicity associated with immunotoxins prepared with whole intact ricin. Specific monoclonal antibodies have been generated against ricin, or ricin chains A or B (8); however, it is not known whether any of these mAbs provide protection against the *in vivo* toxicity of ricin.

This manuscript describes the generation and characterization of a murine anti-ricin IgG_1 mAb, termed BG11-G2. Its specificity was demonstrated by binding to intact ricin, but not to unrelated toxins such as TDT, STX, and T-2 mycotoxin. BG11-G2 mAb reacted with an antigenic determinant associated with the intact ricin molecule, since it did not bind to purified ricin A or B chains, nor were the purified chains capable of inhibiting the binding of BG11-G2 mAb to ricin-coated wells.

BG11-G2 anti-ricin mAb was quite effective in protecting EL-4 cells against the *in vitro* toxicity of ricin as determined by the EL-4 cell assay. This observation led us to attempt to passively protect BALB/c mice against the *in vivo* toxicity of ricin with purified BG11-G2 mAb. In a preliminary experiment, it was determined that normal BALB/c mice injected intraperitoneally with 1.0 ml of BG11-G2 ascites exhibited optimum levels of circulating mAb approximately 18 hr post-infusion (data not shown). This mAb level persists for 24 to 48 hr, and slowly declines thereafter. Although a single injection of BG11-G2 mAb given approximately 18 hr prior to ricin challenge considerably delayed the onset of death, all mice eventually died. Thus, passive treatment with anti-ricin mAb before ricin challenge appeared to reduce the toxic levels of ricin in the circulation. Significantly better protection against ricin toxicity was achieved when BG11-G2 mAb was given prior to and after ricin challenge. Monoclonal antibody-treated mice challenged with 2.0 μg of ricin were completely protected against ricin *in vivo* toxicity. Attempts are underway to produce murine monoclonal and rabbit polyclonal anti-idiotypic antibodies exhibiting antigenic mimicry (16,17) which may serve as effective vaccines in inducing an active and protective anti-ricin antibody response.

ACKNOWLEDGEMENTS

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Appendix F

Immunology 1993 79 681-684

BRIEF COMMUNICATION

Polyclonal anti-idiotypes induce antibody responses protective against ricin cytotoxicity

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SUMMARY

Protein G-purified goat anti-ricin IgG, previously demonstrated to protect against ricin toxicity *in vitro* and *in vivo*, was used to raise BALB/c mouse and New Zealand White rabbit polyclonal anti-idiotypic antibodies. The generated anti-idiotypic sera were repeatedly absorbed over agarose conjugated to normal goat immunoglobulins, and purified by protein A–agarose affinity chromatography. Immunization of BALB/c mice with BALB/c anti-idiotypes did not result in a significant anti-ricin antibody response. However, injection of BALB/c mice with BALB/c anti-idiotypes conjugated to keyhole limpet haemocyanin (KLH) or with unconjugated rabbit anti-idiotypes resulted in specific and anti-ricin immune responses. The anti-idiotype-induced anti-ricin antibody responses protected against the *in vitro* cytotoxicity of ricin, a potent plant-derived protein synthesis inhibitor, as assessed by the murine EL-4 leukaemia cell assays. Thus, anti-idiotype-based vaccines may represent an alternative, safe and effective means of inducing protective immunity against toxins such as ricin, whose extreme *in vivo* toxicity render them unsafe as immunogens.

The network hypothesis as formulated by Jerne envisions the immune system as a complex and interacting network of lymphocytes mediated by complementary structures encoded by immunoglobulin variable (V) region genes.[1] Idiotypes (Id) are defined as antigenic determinants or amino acid sequences associated with the V region of an antibody molecule. One of the postulates of Jerne's hypothesis is that 'internal image' anti-Id antibodies can mimic the tertiary conformation of the original antigen and, thereby, interact with the immune system to elicit specific responsiveness. Thus, anti-Id capable of serologically mimicking the nominal antigen may have applications in vaccine development strategy.[2] The ability of anti-Id to modulate the *in vivo* immune responsiveness to a variety of viral, bacterial, and parasitic infections has been demonstrated.[3-5] We have previously demonstrated that a murine monoclonal anti-Id antibody raised against a monoclonal antibody (mAb) specific for T-2 mycotoxin, a protein synthesis inhibitor, was capable of inducing in syngeneic mice a systemic and protective immune response against the *in vitro* and *in vivo* toxicity of T-2.[5,6] Recently, rabbit and BALB/c mouse polyclonal anti-Id specific for burro anti-saxitoxin (anti-STX) IgG were shown by us to induce a specific antibody response in naive BALB/c mice against STX, a potent sodium channel blocker (T. Chanh *et al.*, manuscript submitted).

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A large variety of plants produce toxic substances which rank among the most poisonous compounds known. Perhaps the most extensively investigated is the protein synthesis inhibitor ricin, isolated from the seeds of the castor plant *Ricinus communis*. Ricin is a disulphide-linked heterodimer consisting of two glycoproteins, A and B chains, of approximately 32,000 MW each.[7] The B chain is a lectin with binding affinity for cell membrane galactosides.[8] Binding to cell-surface receptors mediated by the B chain triggers the endocytic uptake of the ricin molecule, and facilitates the intracellular escape of the A chain from endosomes into the cytoplasm.[9] The ricin A chain carries the toxic moiety which enzymatically attacks the 60S ribosomal subunit and disrupts cellular protein synthesis.[10]

As a result of its extreme *in vivo* toxicity, ricin cannot be used as the immunogen to elicit protective immunity. To circumvent this problem, polyclonal anti-Id raised against a protein G-purified goat anti-ricin IgG preparation, previously shown to protect against ricin toxicity,[11,12] was administered into naive BALB/c mice and was shown to elicit a specific anti-ricin antibody response which protected against ricin *in vitro* cytotoxicity, as determined by EL-4 murine leukaemia cell assays. These experimental results reinforce the concept of the anti-Id-based approach for vaccine development, especially against biological and chemical toxins whose low molecular weights, chemical nature, and extreme *in vivo* toxicity do not allow their use as safe immunogens.

Polyclonal anti-Id sera were generated by immunization of BALB/c mice and New Zealand White rabbits intraperitoneally

Table 1. ELISA reactivity of sera from BALB c mice immunized with KLH-BALB c anti-goat (anti-ricin)

Mouse designation	Immunizations									
	3	4			5			6		
	Ig*	Ig	μ*	γ*	Ig	μ	γ	Ig	μ	γ
0.0	640†	640	ND‡	10	1280	320	ND	5120	2560	640
0.1	640	640	ND	20	1280	640	ND	2560	1280	640
1.0	1280	1280	ND	10	2560	1280	ND	5120	2560	80
0.2	1280	1280	ND	20	5120	2560	ND	5120	2560	1280
2.0	1280	1280	ND	20	1280	1280	ND	5120	2560	1280

* Goat anti-mouse immunoglobulins, anti-μ-chain and anti-γ-chain were used respectively as secondary reagents.

† Reciprocal dilution of sera considered positive (OD > 3 × OD of preimmune).

‡ Not determined.

Table 2. ELISA reactivity of sera from BALB/c mice immunized with rabbit anti-goat (anti-ricin)

Mouse designation	Immunizations									
	2	3	4			5			6		
	Ig*	Ig	Ig	μ*	γ*	Ig	μ	γ	Ig	μ	γ
0.0	80*	1280	1280	640	10	2560	2560	20	5120	5120	10
0.1	80	1280	2560	1280	40	5120	2560	320	10,240	5120	640
1.0	10	640	2560	1280	20	5120	5120	40	5120	5120	320
0.2	320	1280	2560	640	80	1280	1280	160	5120	2560	320
2.0	640	640	2560	1280	160	2560	2560	160	10,240	5120	320

* Goat anti-mouse immunoglobulins, anti-μ-chain and anti-γ-chain were used respectively as secondary reagents.

(i.p.) and intramuscularly (i.m.), respectively, with 10 μg/mouse or 50 μg/rabbit of protein G-purified goat anti-ricin IgG precipitated in aluminum hydroxide prepared as previously described.[13] Anti-isotype and anti-allotype reactivities in the polyclonal anti-Id sera were removed by repeated absorptions with agarose normal goat immunoglobulin (NGIg) affinity chromatography (Sigma Chemical Co., St Louis, MO) as previously detailed[13] and checked for lack of reactivity against NGIg by enzyme-linked immunosorbent assay (ELISA).[6] Absorbed murine or rabbit anti-Id sera reactive with goat anti-ricin IgG, but not with NGIg, were further purified by protein A affinity chromatography and precipitated with aluminum hydroxide.[13] Groups of five BALB/c mice were immunized with 10 μg/mouse of alum-precipitated murine or rabbit anti-Id i.p. every other week, with bleedings obtained before each injection. The purified murine anti-Id antibodies were also conjugated to keyhole limpet haemocyanin (KLH; Calbiochem, San Diego, CA) by the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC; Sigma Chemical Co.) procedure.[6,13] Other groups of five BALB/c mice were immunized with syngeneic anti-Id–KLH in alum, as described above. Sera obtained after each immunization were tested for reactivity against ricin, and those reactive with ricin were tested for binding to the irrelevant neurotoxin STX[14] and for protection against ricin cytotoxicity using the EL-4 cell line as described previously.[15] Mice immunized with mouse anti-Id as alum precipitate did not develop significant anti-ricin reactivity even after five immunizations (data not shown). On the other hand, BALB/c mice immunized with syngeneic anti-Id–KLH exhibited detectable anti-ricin antibodies following the third immunization (Table 1). Mice immunized with rabbit anti-Id also developed detectable anti-ricin reactivity following the second immunization (Table 2). The anti-ricin titres in both groups of mice increased after subsequent immunizations of anti-Id. The anti-ricin IgG titres ranged from 1:2560 to 1:5120 and from 1:5120 to 10,240 in mice immunized with syngeneic and rabbit anti-Id, respectively. The predominant isotype in the anti-ricin responses in both groups of mice was IgM. The anti-ricin titres detected with specific anti-mouse μ-chain were between 1:1280 and 1:5120, whereas those detected with specific anti-mouse γ-chain ranged between 1:80 and 1:1280, following the sixth immunization. The anti-Id-induced anti-ricin immune responses were specific in that significant reactivity was obtained with purified ricin chains A- and B-coated microtitre wells, whereas no binding was observed with the irrelevant STX (data not shown).

The ability of the anti-Id-induced anti-ricin antibody responses to protect against *in vitro* toxicity of ricin was assessed using the murine EL-4 leukaemia cell assay.[15] Results of a

Table 3. Anti-Id-induced anti-ricin antibody responses protect against ricin cytotoxicity

Pooled mouse sera/dilution	Without ricin	With ricin*	%I†	%P†
None	65,980 ± 6234‡	23,167 ± 3015	64·9	
Preimmune	61,978 ± 5923	22,768 ± 2843	63·3	
Mouse anti-Id control (10)§	51,642 ± 5015	19,814 ± 2101	61·6	
Rabbit anti-Id control (10)§	49,156 ± 4811	21,249 ± 1958	56·8	
Mouse anti-Id immune				
10	59,079 ± 6,02	62,152 ± 5384	0	100·0
50	63,183 ± 6623	58,923 ± 6013	6·7	89·4
100	66,287 ± 7265	43,164 ± 5028	34·9	44·9
200	59,274 ± 6292	28,425 ± 3102	52·0	17·9
Rabbit anti-Id immune				
10	67,201 ± 7017	65,918 ± 7016	1·9	97·0
50	66,871 ± 6901	55,926 ± 6127	16·4	74·1
100	69,021 ± 9170	42,635 ± 4561	38·2	39·7
200	64,102 ± 6391	29,156 ± 4120	54·5	13·9

* The final concentration of ricin used was 6·25 ng/ml.

† Per cent inhibition (%I) and per cent protection (%P) were calculated as described in the text.

‡ Mean ± SD c.p.m. of triplicate determinations.

§ Sera from mice immunized with an anti-Id mAb-KLH[6] and polyclonal rabbit anti-Id[16] against an anti-T-2 mAb1, respectively.

representative experiment are shown in Table 3. Pooled sera from both anti-Id-immune mouse groups provided significant protection against ricin toxicity on EL-4 cells. Pooled sera from either group of mice at 1:10 dilution completely protected (100% and 97·0% protection) EL-4 cells against ricin toxicity. At a dilution of 1:100 of the pooled Ab3 sera from mice immunized with syngeneic anti-Id-KLH, approximately 34·9% EL-4 cytotoxicity was obtained as compared to 63·3% toxicity observed in the presence of preimmune system. Thus, this dilution of mouse Ab3 sera protected against ricin cytotoxicity by approximately 44·9%. Likewise, pooled sera of mice immunized with rabbit anti-Id at a dilution of 1:100 provided approximately 39·7% protection against ricin toxicity. Protection against ricin cytotoxicity by sera of both mouse groups was dose dependent. Pooled sera (1:10) from preimmune mice and from mice similarly immunized with a BALB/c anti-Id monoclonal antibody (mAb)-KLH,[6] or with rabbit polyclonal anti-Id[16] against an anti-T-2 mycotoxin mAb1 did not exhibit anti-ricin antibody reactivity in ELISA, and did not protect against ricin toxicity of EL-4 cells (Table 3).

Herein, experimental evidence is presented for the induction in BALB/c mice of a specific and protective anti-ricin immune response by rabbit and BALB/c mouse polyclonal anti-Id specific for a goat anti-ricin IgG preparation. Whereas rabbit anti-Id not conjugated to the protein carrier KLH was efficient in eliciting a specific anti-ricin antibody response in mice, BALB/c mouse anti-Id required conjugation to KLH. The requirement of protein carrier conjugation for the induction of anti-antigen responses in syngeneic systems is in agreement with previously published results,[17] including our studies of anti-mycotoxin T-2[6] and anti-STX (T. C. Chanh and J. F. Hewetson, unpublished data) antibody responses induced by syngeneic mouse anti-Id. In general, the titres of anti-ricin Ab3 responses induced by rabbit anti-Id and by mouse anti-Id-KLH appeared to be comparable. Whereas anti-ricin reactivity was not detectable until after three immunizations with the mouse anti-Id-KLH, low but detectable reactivity was observed following two immunizations with rabbit anti-Id. It is noteworthy that the predominant isotype of anti-ricin antibody responses induced by both anti-Id preparations injected as aluminum hydroxide was of the IgM isotype, whereas the anti-KLH IgG antibody titres in mice immunized with syngeneic anti-Id-KLH ranged from 1:100,000 to 1:200,000. It would be of interest to determine whether immunization with anti-Id admixed in other adjuvant systems would result in induction of a higher anti-ricin IgG response.

The generation of anti-Id against polyclonal anti-ricin Ab1 of a different species, capable of eliciting specific Ab3 responses is suggestive of the presence of 'internal image' anti-Id, and represents the first demonstration of the induction of specific and protective anti-ricin immune responses by polyclonal anti-Id reagents. These results provide justification for further investigations of anti-Id-based vaccines for the generation of systemic and protective immunity against toxins of biological and chemical origins, whose extreme toxicity prevents their use as safe conventional immunogens.

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